ALKALINE LIPASE PRODUCTION BY MARINE BACILLUS SMITHII BTM S 11

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by

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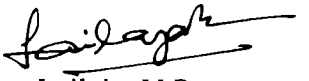
CERTIFICATE

This is to certify that the research work presented in the thesis entitled "**Alkaline Lipase Production by Marine *Bacillus smithii* BTMS 11**" is based on the original research work carried out by Ms. Lailaja.V.P 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 "**Alkaline Lipase Production by Marine *Bacillus smithii* BTMS 11**" 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 in Biotechnology and the thesis or no part thereof has been presented for the award of any degree, diploma, associate ship or other similar titles or recognition.



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Lailaja. V.P

****Dedicated to my family*

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"Technology, unlike Science is a group activity. It does not grow on individual intelligence, but by intelligences interacting and ceaselessly influencing one another". These words of A P J Abdul Kalam falls right at the juncture when this thesis becomes fruitful with the collective efforts of interacting thoughts.

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ABBREVIATIONS

%	-	Percentage
°C	-	Degree Celsius
µ	-	micron
µg	-	microgram
µl	-	microlitre
µM	-	micro Molar
A_{280}	-	Absorbance at 280 nm
AIDS	-	Acqiured Immuno Deficiency Syndrome
ANOVA	-	Analysis of Variance
APS	-	Ammonium persulphate
BLAST	-	Basic Local Alingment search Tool
bp	-	Base pairs
BSA	-	Bovine Serum Albumin
cfu	-	colony forming unit
cm	-	centimetre
DEAE Cellulose	-	Diethyl amino ethyl cellulose
DNA	-	Deoxyribonucleic Acid
dNTP	-	Deoxy ribo nucleic acid
DW	-	Distilled water
EC	-	Enzyme Classification
EDTA	-	Ethylene diamine tetra acetic acid
g	-	grams
hrs	-	hours
kDa	-	kilo Dalton
K_m	-	substrate concentration at which the reaction velocity is half maximum
kV	-	Kilo Volt

LB	-	Luria broth
M	-	Molar
(M_r)	-	Relative molecular weight
mg	-	milligram
ml	-	millilitre
mm	-	millimeter
MUF	-	4-Methylumbelliferyl
mM	-	milli Molar
MW	-	molecular weight
NJ	-	Neighbor joining
nm	-	nanometer
O D	-	optical density
PAGE	-	Polyacrylamide gel electrophoresis
PEG	-	Polyethylene glycol
pNP	-	Para nitro phenyl -
rpm	-	rotations per minute
RT	-	Room Temperature
SDS	-	Sodium dodecyl sulphate
SmF	-	Submerged Fermentation
sp.	-	Species
SmF	-	Submerged Fermentation
TCA	-	Trichloro acetic acid
TEMED	-	N-N-N'-N'-Tetramethyl ethylene diamine
Taq	-	*Thermus aquaticus*
TLC	-	Thin Layer Chromatogaphy
U/mg	-	Units/ milligram
U/ml	-	Units / milliliter
UV	-	Ultra violet
V_{max}	-	maximal velocity

v/v	-	volume / volume
w/v	-	weight/ volume

CONTENTS

Chapter 1

INTRODUCTION

1.1 GENERAL 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. The majority of the industrial enzymes are of microbial origin. Until the 1960s, the total sales of enzymes were only a few million dollars annually, but the market has since grown spectacularly. According to a report from Business Communications Company, Inc. the global market for industrial enzymes was estimated at $2 billion in 2004. Volume growth of industrial enzymes is between 4% and 5% AAGR (average annual growth rate), which is accompanied by decreasing prices, due to the increase in the number of smaller players competing in the market. As a result, the market is expected to rise at an AAGR of a little over 3% over the next 4 years, and the total industrial enzyme market in 2009 is expected to reach nearly $2.4 billion (Rajan, 2004). 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 actionThe industrial enzyme market is divided into three application segments: technical enzymes, food enzymes, and animal feed enzymes (Fig: 1.1). Protease and amylase lead the market with current shares of 25% and 20%, respectively.

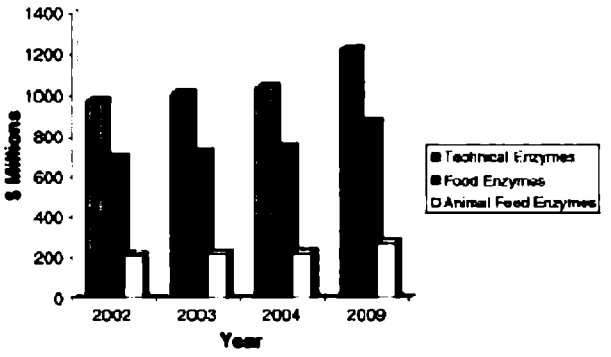


Fig: 1.1 Global enzyme markets by application sectors, through 2009 ($ millions).

It is only in the last decade that lipases have gained importance to a certain extent over proteases and amylases especially in the area of organic synthesis. Now lipases have emerged as key enzymes in swiftly growing biotechnology owing to their multifaceted properties, which find usage in a wide array of industrial applications, such as food technology, detergent, chemical industry and biomedical sciences (Jaeger *et al*, 1994, 1999). 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).

Lipolytic enzymes catalyzing the conversion of lipids comprise lipases (triacylglycerol hydrolases) [EC 3.1.1.3], esterases [EC 3.1.1.1], phospholipases [EC 3.1.4.3] and lysophospholipases (different registrations [EC 3.1.x.x]) (IUBMB, 1992; Bairoch, 1999).Lipases generally called as triacylglycerol hydrolases, are serine hydrolases and are an important group of biotechnologically relevant enzymes.

Lipases are available from plant animal and microbial sources but those produced from microbes and specifically bacterial lipases play a vital role in commercial ventures. Lipase-producing bacterial genera basically include Bacillus, Pseudomonas and Burkholderia.

Lipases research gained momentum only 100 years ago when a landmark report was published by Eijkmann (1901) describing lipolytic activity by different bacteria on beef tallow spread on the ground of a glass plate was overlaid with agar. Today lipases are the biocatalyst of choice and this fact is prominently evident from the hundreds of research papers which are coming to limelight bringing forth various aspects of this enzyme.

Lipases from a large number of bacterial, fungal and plant and animal sources have been purified to homogeneity (Saxena *et al*,2003).Lipases isolated from different sources have a wide range of properties depending on their sources with respect to positional specificity, fatty acid specificity, thermostability, pH optimum, etc. (Huang, 1984).

Microbial lipases are produced mostly by submerged culture (Ito *et al.*, 2001), but solid state fermentation methods (Chisti, 1999a) can also be used. Immobilized cell culture has been used in a few cases (Hemachander *et al.*, 2001). Many studies were undertaken to define the optimal culture and nutritional requirements for lipase production by submerged culture. Lipase production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature, and the dissolved oxygen concentration (Elibol and Ozer, 2001). Lipidic carbon sources seem to be generally essential for obtaining a high lipase yield; however, a few studies

produced good yields in the absence of fats and oils. Most lipases can act in a wide range of pH and temperature, though alkaline bacterial lipases are more common and are always in high demand in the industrial sector. Because of their high specificity, stability in organic solvents, chemoselectivity, regioselectivity and enantioselectivity, lipases have gained a distinguished platform contrary to its contemporary hydrolases especially in the area of organic synthesis. Moreover with the advent of modern molecular approaches like recombinant technology, site directed mutagenesis and directed evolution, the optimisation of industrially relevant lipase properties has become feasible and has added wheels to the lipase research and paved way for the development of novel and improved lipases. Novel biotechnological applications have been successfully established using lipases for the synthesis of biopolymers, biodiesel, and production of enantiopure pharmaceuticals, agrochemicals and flavour compounds.

In a nutshell, with its renowned and distinct kinetic action, lipases have percolated into almost all overriding industrial arena thereby making them inevitable biocatalysts of choice against chemical synthesis. Alkali tolerance and thermostability of lipase are the most desirable characteristics for their commercial exploitation. Although the productions of such lipases have been reported but the venture for new and improved lipases are still in the pipeline (Boreto *et al*, 1997; Neelima *et al*, 1999). The continuous demand for highly active enzymes with appropriate properties and substrate specificities encourages the search for new enzyme sources. The search for new lipase/esterase sources is of interest, because it could provide new biocatalysts that could either promote novel industrial applications, or be used to achieve a better understanding of enzymes mechanism and structure–function relationships.

But so far the lipases reported are from the terrestrial origin, and the marine environment that holds the vast diversity of flora and fauna remains under explored in search of novel enzymes.

For the last two decades the common conclusions of international flora considering strategic challenges in science have uniformly identified the marine biotope as a large and untapped area for exploration (ESF-Marine Board, 2001). The great metabolic diversity of marine microbes makes the oceans a rich source of biological material for biotechnology applications. A variety of secondary metabolites, enzymes, polymers, and metabolic processes can be found only in the microbes that inhabit the oceans, and it is likely that many more useful products will be found as research continues. The biotechnology potential of these organisms is a major driving force behind the push to characterize marine microbial diversity. Different marine habitats will yield microbes suited to different applications. Hence in the present study marine bacteria was desired as the target organism as a source of lipase.

1.2 SPECIFIC OBJECTIVE OF THE PRESENT STUDY INCLUDES THE FOLLOWING:

Considering the potential of marine environment present study was designed for the screening and isolation of a potential salt tolerant, alkaline and thermotolerant lipase producing bacteria from the costal belts of South India and consequent development of ideal bioprocess for industrial production, purification characterisation and evaluation of the potential of the lipase enzyme for various industrial applications.

1. Screening and isolation of a potential lipase producing bacteria.
2. Optimization of various physicochemical factors in Submerged fermentation for the production of alkaline lipase
3. Purification of the lipase enzyme
4. Characterisation of the enzyme
5. Evaluation of the enzyme for various industrial applications

Chapter 2

REVIEW OF LITERATURE

Lipases research gained momentum only 100 years ago when a landmark report was published by Eijkmann (Eijkmann.,1901) describing lipolytic activity by different bacteria on beef tallow spread on the ground of a glass plate was overlaid with agar.

A 'true' lipase (EC 3.1.1.3) is defined as a carboxylesterase, which catalyses the hydrolysis and synthesis of long-chain acylglycerols with trioleoylglycerol being the standard substrate (Gerhartz., 1990).

Lipases belong to the class of serine hydrolases and therefore do not require any cofactor for reaction. 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. But 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 (Saxena *et al.*, 1999).A typical reaction catalyzed by lipase is shown in Figure 2.1.

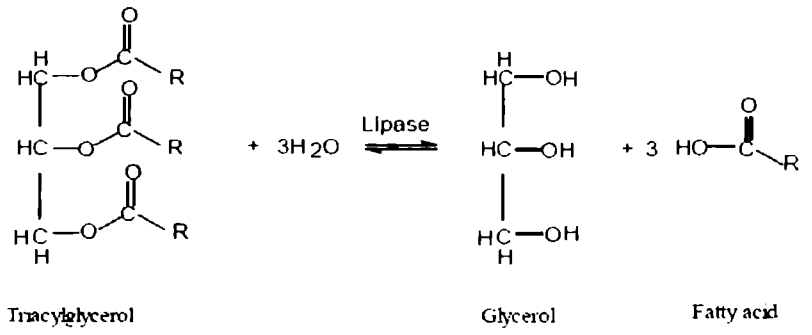


Fig: 2.1 Lipase catalysed reaction

2.1 Source and occurrence of lipases:

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 *et al*, 1985). Many bacteria are found to prefer neutral pH but there are reports of alkalophilic (Gao *et al.*, 2000.; Ghanem *et al.*, 2000) bacteria also. Psychrophilic and thermophilic organisms, as well as organisms having different oxygen demand (aerobic, microaerophilic and anaerobic) are reported to produce lipases. lthough a number of lipase-producing bacterial sources are available, only a few are commercially exploited as wild or recombinant strains (Jaeger *et al.*, 1994; Palekar *et al.*, 2000). Of these, the important ones are: *Achromobacter sp*, *Alcaligenes sp*, *Arthrobacter sp*, *Bacillus sp*, *Burkholderia sp*, *Chromobacterium sp* and *Pseudomonas sp*. Of these, the lipases from *Pseudomonas* bacteria are widely used for a variety of biotechnological applications (Jaeger *et al.* 1994; Pandey *et al.* 1999; Beisson *et al.* 2000). Table 2.1 summarizes the large number of bacteria, *actinomycetes* that produces lipases.

Table: 2.1 Lipase producing Bacteria and Actinomycetes

Bacteria		**References**
Acinetobacter	*Acinetobacter sp*	Wakelik and Forster 1997; Barbaro *et al*, 2001
	Aci. Pseudoalkaligens	Sztajer *et al.*, 1998
	Aci. Radioresistens	Chen *et al.*, 1999; Liu and Tsai ,2003
	Aci. Calcoaceticus	Dharmsthiti *et al.*, 1998; Jaeger *et al*, 1999., Pandey *et al*, 1999., Pratuangdejkul and Dharmsthiti, 2000
	Acinetobacter sp SY-01	Soo-Jin Han , 2004
Aeromonas	*Ae.hydrophila*	Anguita *et al*, 1993
	Ae. sorbia LP004	Lotrakul and Dharmsthiti, 1997
Achromobacter	*Achromobacter sp*	Mitsuda *et al*,1988
	A. lipolyticum	Brune and Gotz, 1992., Davranov 1994
Alcaligenes	*Alcaligenes sp*	Mitsuda *et al*, 1988
	A.denitrificans	Odera *et al*, 1986
Arthrobacter	*Arthrobacter sp.*	Pandey *et al*, 1999
Archaeglobus	*A.fulgidus*	Jaeger *et al* , 1999

	B.megaterium	Hirohara *et al.*, 1985
	B.cereus	El-Shafei and Rezkallah, 1997
	B.stearothermophilus	Kim *et al.*, 1998 ; Bradoo *et al*, 1999; Jaeger *et al.*, 1999; Sangpill *et al.*,2004.
	B.subtilis	Kennedy and Rennarz 1979 Jaeger *et al*, 1999
	Recombinant B.subtilis 168	Lesuisse *et al.*1993
	B.brevis	Hou 1994
	B.thermocatenulatus	Rua *et al*, 1998 ., Jaeger *et al*, 1999., Pandey *et al*, 1999.,Nils et al, 2004
Bacillus	*Bacillus sp. IHI-91*	Becker *et al*, 1997
	Bacillus strain WAI 28A5	Janssen *et al*, 1994
	Bacillus sp	Helisto and Korpela 1998., Pandey *et al.* 1999 ., Sharma *et al*, 2002a.,Burku and Kubilay, 2006
	B.coagulans	Kanwar *et al*,2006. Satyendra *et al*,2005
	B. acidocaldarius	Manco *et al.*, 1998
	Bacillus sp RS-12	Sidhu *et al.*, 1998a,b
	B.thermooeovorans ID-1	Lee *et al.*, 1999
	B.thermooeovorans	Lelie *et al*,2005
	Bacillus sp J33	Nawani and Kaur., 2000
	B. alcalophillus	Ghanem *et al.*, 2000
	B. atrophaeus	Bradoo *et al.*, 1999
	B.laterosporus	Toyo-Jozo ,1988
	B. pumilus	Jaeger *et al*, 1999
	B.sphaericus	Toyo-Jozo 1988, Chin John Hun *et al*, 2003
	B.thaiminolyticus	Toyo-Jozo, 1988
Brochothris	*B.thermosphacta*	Brune and Gotz , 1992
Burkholderia	*Burkholderia sp.*	Yeo *et al*, 1998.
	B.glumae	Jaeger and Reetz., 1998 Reetz and Jaeger., 1998
Chromobacterium	*C.violaceum*	Koritala *et al.*, 1987
	C.viscosum	Jaeger and Reetz., 1998 Jaeger *et al*, 1999 Helisto and Korpela .,1998

Cryptococcus	*C.laurentii*	Toyo-Jozo 1988;
Enterococcus	*E.faecalis*	Kar *et al.*, 1996
	L.delbruckii sub sp.bulgaricus	El-Sawah *et al.*, 1995
Lactobacillus		
	Lactobacillus sp	Meyers *et al* ., 1996
	L.cuvantus	Brune and Gotz (1992)
	L.plantarum	Lopes Mde *et al.*, 2002
	Lactobacillus sp.	Meyers *et al.*, 1996
Micrococcus	*M.freudenreichii*	Hou 1994
	M.luteus	Hou 1994
Morexella	*Morexella sp*	Jaeger *et al.*, 1999
Mycobacterium	*M.chelonae*	Pandey *et al.*, 1999
Propionibacterium	*P.acne*	Sztajer *et al.*, 1988
	P.granulosum	Sztajer *et al.*, 1988
	P.aeruginosa	Hou., 1994;Ito *et al.*, 2001.
	P.fragi	Jaeger *et al.*, 1994, Schuepp *et al.*, 1997, Granem *et al.*, 2000.
	P.mendocina	Jaeger and Reetz., 1998,
Pseudomonas		Jaeger et al ., 1999, Surinenaite *et al.*, 2000.
	P.putida 3SK	Lee and Rhee., 1993
	P. glumae	Frenken *et al.*, 1993,
	P.cepacia	Pencreac'h and Barati., 1996., Lang *et al* 1998, Hsu *et al* 2000.
	P.fluorescens	Maragoni 1994, Arpigny and Jaeger 1999, Pandey *et al* 1999.
	P.aeruginosa KKA-5	Sharon *et al* .,1998
	P.pseudoalkaligens F-11	Lin *et al* 1995, 1996.
	Pseudomonas sp	Sin *et al.*, 1998, Reets and Jaeger., 1998, Dong *et al* .,1999.
	Pseudomonas sp KW156	Yang *et al* .,2000
	P.aureufacienus	Koritala *et al* .,1987
	P.luteola	Arpgny and Jaeger 1999, Litthauer *et al.*, 2002
	P.nitroreducens var thermotolerrance	Ghanem *et al* .,2000
	P.pseudomallei	Kanwar and Goswami 2002

	P.wisconsinensis	Arpigny and Jaeger., 1999
Pasteurella	*P.multocida*	Pratt *et al.*, 2000
Propionibacterium	*P.acnes*	Jaeger *et al.*, 1999
	P.avidum	Brune and Gotz .,1992
	P.granulosum	Brune and Gotz .,1992
Proteus	*P.vulgris*	Jaeger *et al.*, 1999
Psycrobacter	*P.immobilis*	Jaeger *et al.*, 1999
Staphylococcus	*S.canosus*	Tahoun *et al* .,1985
	S.aureus	Simons *et al.*, 1996; Jaeger *et al.*,1999.
	S.hyicus	Van Oort *et al.*, 1989., Meens *et al*, 1997., Van Kampen *et al* , 1998, 2001 Jaeger *et al* , 1999.
	S.epidermis	Simons *et al* ., 1998 Jaeger *et al.*, 1999.
	S.warnari	Pandey *et al.*, 1999.,.
	S.haemolyticus	Oh *et al.*, 1999.
	S.xylosus	Pandey *et al.*, 1999., Van Kampen *et al* ., 2001
Streptococcus	*S.lactis*	Sztajer *et al.*, 1988.
Serratia	*S.maecescens*	Matsumae *et al.*, 1993, 1994., Pandey *et al.*, 1999; Abduo, 2003
Streptomyces	*S.exfoliatus*	Arpigny and Jaeger 1999
Sulfobus	*S.acidoclarius*	Jaeger *et al* .,1999
Vibrio	*V.cholerae*	Jaeger *et al.*, 1999

2.2 Lipase Production

Bacterial lipases are mostly extracellular and are greatly influenced by nutritional and physico-chemical factors, such as temperature, pH, nitrogen and carbon sources, presence of lipids, inorganic salts, agitation and dissolved oxygen concentration (Jaeger *et al.*, 1994; Kim *et al.*, 1996).

Bacterial lipases are mostly inducible enzymes, requiring some form of oil, fatty acid, fatty acid alcohol or fatty acid ester for induction. However, there are a few reports of constitutive lipase production by bacteria. (Gao *et al.*, 2000). Lipases are usually secreted out in the culture medium; although there are a few reports of the presence of intracellular lipases (Lee and Lee., 1989) as well as cell bound lipases (Large *et al.*, 1999).The major factor for the expression of lipase activity has always been carbon, since lipases are by and large inducible enzymes (Lotti *et al.*,, 1998) and are thus generally produced in the presence of a lipid source such as an oil or any other inducer, such as triacylglycerols, fatty acids, hydrolysable esters, tweens, bile salts and glycerol (Bradoo *et al.*,. 1999; Rathi *et al.*, 2001). However, their production is significantly influenced by other carbon sources, such as sugars, sugar alcohol, polysaccharides, whey, casamino acids and other complex sources (Ghanem *et al.*, 2000). Certain long-chain fatty acids, such as oleic, linoleic and linolenic acids, are known to support lipase production from various bacteria, such as P. mephitica (Ghosh *et al.* 1996).

Besides carbon source, the type of nitrogen source in the medium also influences the lipase titers in production broth (Ghosh *et al.* 1996). Generally, organic nitrogen is preferred, such as peptone and yeast extract, which have been used as nitrogen source for lipase production by various *Bacillus spp* (viz. *Bacillus* strain A30-1, B. *alcalophilus, B. licheniformis* strain H1) and various pseudomonads

(viz. *Pseudomonas sp., P. fragi, P. fluorescens* BW 96CC), *Staphylococcus haemolyticus*; (Wang *et al.*, 1995; Pabai *et al.*, 1996; Oh *et al.*, 1999., Ghanem *et al.*, 2000; Lanser *et al.*, 2002; Sharma *et al.*, 2002b), while tryptone and yeast extract have been used in the case of *S. haemolyticus* L62 (Oh *et al.*, 1999). Inorganic nitrogen sources such as ammonium chloride and diammonium hydrogen phosphate have also been reported to be effective in some microbes (Dong *et al.*, 1999; Rathi *et al.*, 2001).

Several researchers have studied the effects of various polysaccharides and detergents on lipase production. Winkler and Stuckmann (1979) have reported that the exolipase production by *Serratia marcescens* was enhanced by polysaccharides like glycogen, hyluronate, laminarin, pectin B and gum Arabic while the exolipase production by *Pseudomonas aeruginosa* was enhanced by treatment with glycogen (Schulte *et al.* 1982), hyaluronate (Jaeger and Winkler 1984) and alginate (Wingender and Winkler 1984). A "detachment hypothesis" has been proposed by Winkler and Stuckmann (1979) suggesting the interaction of polysaccharides with cell surface structures as probable mechanism of this phenomenon. They have suggested the presence of hypothetical binding sites for newly synthesized lipase. The polysaccharides were suggested to interact with these lipase molecules by either competition for these sites or by changing the conformation of exolipase.

Cofactors are generally not required for lipase activity, but divalent cations such as calcium often stimulate enzyme activity. Divalent cations stimulate or inhibit enzyme production in microorganisms. Rathi *et al.* (2001) observed stimulation in lipase production from *Burkholderia* sp. in the presence of Ca^{2+} and Mg^{2+}. Sharma *et al.* (2002) reported stimulation in lipase production from *Bacillus sp.*

RSJ1 in the presence of calcium chloride. However, most other metal ion salts were inhibitory to lipase production. This has been suggested to be due to the formation of the calcium salts of long-chain fatty acids (Godtfredsen., 1990). Calcium- stimulated lipases have been reported in the case of *B. subtilis* 168 (Lesuisse *et al.*, 1993), *B. thermoleovorans* ID-I (Lee *et al.*, 1999), Further, lipase activity is in general inhibited drastically by heavy metals like Co^{2+}, Ni $^{2+}$, $Hg^{2+}$and $Sn^{2+}$and slightly inhibited by Zn^{2+} and Mg^{2+} (Patkar and Bjorkling, 1994).However, the lipase from *A. calcoaceticus* LP009 was stimulated by the presence of Fe3+ and its activity was reduced by less than 20% on addition of various other ions (Dharmsthiti *et al.*, 1998). Iron was found to play a critical role in the production of lipase by Pseudomonas sp. G6 (Kanwar *et al.*, 2002).

In addition to the various chemical constituents of a production medium, physiological parameters such as pH, temperature, agitation, aeration and incubation period also play an important role in influencing production by different microorganisms.

Generally, bacterial lipases have neutral (Dharmsthiti *et al.*,1998; Dharmsthiti and Luchai 1999; Lee *et al.*, 1999) or alkaline pH optima (Kanwar and Goswami 2002; Sunna *et al.* 2002), with the exception of *P. fluorescens* SIK W1 lipase, which has an acidic optimum at pH 4.8 (Andersson *et al.*, 1979). Lipases from *Bacillus stearothermophilus* SB-1, *B. atrophaeus* SB-2 *and B. licheniformis* SB-3 are active over a broad pH range (pH 3–12) Bradoo *et* al., 1999). Bacterial lipases possess stability over a wide range, from pH 4 to pH 11 (Dong *et al.*, 1999).The initial pH of the growth medium is important for lipase production. The pH of the medium is generally maintained around 7.0. for best growth and lipase production, such as

in the case of *Bacillus sp.* (Sugihara *et al.* 1991), *Acinetobacter sp.* (Barbaro *et al.* 2001) and *Burkholderia sp.* (Rathi *et al.* 2001). However, maximum activity at higher pH (>7.0) has been observed in many cases (Wang *et al.* 1995., Dong et al., 1999., Sharma *et al.* 2002). Lipases from *Bacillus subtilis* and *B. pumilis* (Moller *et al*, 1991) reported earlier showed optimal activity and stability at extreme alkaline pH values 9.5 (Nthangeni, 2001). A pH range between 8.0-10.0 has been used for lipase production by alkalophillic bacteria.

The optimum temperature for lipase production corresponds with the growth temperature of the respective microorganism. For example, the best temperature for growth and lipase production in the case of *Bacillus* sp. RSJ1 was 50°C (Sharma *et al.*, 2002). Bacterial lipases generally have temperature optima in the range 30–60°C (Litthauer *et al.*, 2002). However, reports exist on bacterial lipases with optima in both lower and higher ranges (Dharmsthiti and Luchai, 1999; Lee *et al.* 1999; Oh *et al.* ,1999; Sunna *et al.*, 2002). Thermal stability data are available only for species of *Bacillus, Chromobacterium, Pseudomonas* and Staphylococcus. The thermostability of the enzyme from *Bacillus sp.* was enhanced by the addition of stabilizers such as ethylene glycol, sorbitol, glycerol, with the enzyme retaining activity at 70°C even after 150 min (Nawani and Kaur., 2000). A few *Pseudomonas* lipases have been reported which are stable at 100°C or even beyond to 150°C with a half-life of a few seconds; (Rathi *et al.*, 2001). A highly thermotolerant lipase has been reported from *B. stearothermophilus*, with a half-life of 15–25 min at 100°C (Bradoo *et al*,. 1999).

The onset of lipase production is organism-specific, but, in general, it is released during late logarithmic or stationary phase (Makhzoum *et al.*, 1995). Fast growing organisms were normally found to secrete the lipase within 12-24 h (Imamura and Kitaura., 2000). Incubation periods ranging from few hours to several days have been found to be best suited for maximum lipase production by bacteria. An incubation period of 12 h was optimum for lipase production by *Bacillus sp.* RSJ1 (Sharma *et al.* 2002) and 16 h for *B. thermocatenulatus* (Schmidt *et al.*, 1997). While maximum lipase was produced after 72 h and 96 h of incubation, respectively, in the case of the *Pseudomonas sp, P. fragi* and *P. fluorescens* BW 96CC (Pabai *et al.*, 1996; Dong *et al.*, 1999).

Most of the fermentations for lipase production have been performed in submerged, batch mode. There are a few reports of fed-batch or continuous culture studies. Lipase production as high as 2000 U/ml was achieved by feeding olive oil. The lipase production was directly coupled with increase in cell mass and reached a value of 230 mg per litre culture supernatant.A list of various fermentation conditions used with different bacteria is presented in Table 2.2

Table: 2.2 physiochemical parameters governing lipase production by different bacteria.

Bacterium /Mixture	pH	Temperature	Incubation Period	Carbon Source	Nitrogen Source	Reference
Acenetobacter sp	7.0	25	9	Tween-80/olive Oil	n.s	Barbaro *et al.*, 2001
A.calcoaceticus	6.8	30	12	Lactic acid/Oleic Acid	n.s	Mahler *et al.*, 2000
A. calcoaceticus LP009	7.0	15	n.s	Tween-80	Tryptone, yeast extract	Pratuangdejkul and Dharmsthiti., 2000
Bacillussp.	7.0	28	80	Olive oil	Peptone, yeast extract	Sugihara *et al.*, 1991
Bacillussp. RSJ1	9.0	50	12	Tween-80/ olive oil	Peptone, yeast extract	Sharma *et al.*, 2002b
Bacillus sp. strain 398	7.2	55	12	Glycerol,	Polypeptone, yeast extract, beef extract	Kim *et al.*, 1994
Bacillus strain A30-1 (ATCC 53841)	9.0	60	15-24	Corn oil	Ammonium chloride, yeast extract	Wang *et al.*, 1995
B. alcalophilus	10.6	60	20	Maltose,	soybean meal Peptone, yeast extract	Ghanem *et al.*, 2000
B. licheniformis strain H1	9.0	50	10	Glucose	Peptone, yeast extract, lab. beef extract	Khyami-Horani., 1996

Table:2.2 (continued)

Burkholderia sp.	7.0	45	24	Glucose, mustard oil	NH4Cl, (NH4)2HPO4	Rathi *et al.*, 2001
Geobacillus sp.	9.0	70	n.s	Tween-80/ olive oil	n.s	Abdel-Fattah., 2002
Pseudomonas sp.	9.0	30	72	Ground soybean, soluble starch	Corn steep liquor, NaNO3	Dong *et al.*, 1999
Pseudomonas sp.	n.s	n.s	60	groundnut oil,	Soya peptone cottonseed meal,	Kulkarni and Gadre 1999
Pseudomonas sp. G6	8.0	34	n.s	n-hexadecane, tributyrin	n.s.	Kanwar *et al.* 2002
Pseudomonas sp. strain KB 700A (recombinant lipase)	7.0	37	16	Casamino acids	Yeast extract	Rashid *et al.*, 2001
P. aeruginosa LP602	7.2	30	48	Whey, soybean oil, glucose	Ammonium sulfate, Yeast extract	Dharmsthiti and Kuhasuntisuk.,1998
P. fragi,P. fluorescens BW 96CCI, P. putida	7.5	30	96	Dextrose, butter	Tryptone, yeast extract	Pabai *et al.*, 1996

Table:2.2 (continued)

P. putida ATCC 795	7.5	27	72	Soybean flour, soluble starch, unsalted butter	Bacto-peptone	Pabai *et al.*, 1995
P. putida 3SK	n.s	30	24	Olive oil	n.s.	
S. haemolyticus 1.62	7.0	37	20	n.s	. Tryptone, yeast extract	Oh *et al.*, 1999
Bacillussp., Pseudomonas sp.	n.s	30	24	Dextrose, triolein	Tryptone, yeast extract	Lee and Rhee., 1994 Lanser *et al.* ,002
Bacillussp., Pseudomonas sp., Arthrobacter sp., Chromobacteriu msp., Staphylococcus sp., Streptococcus sp	n.s	28	5 days	Glucose, soybean oil	Asparagine	Koritala et al., 1987
P. aeruginosa	8.5	37	6	Tween-80	KNO_3	Gilbert *et al.*, 1991
Bacillus sphaericus 205y	7.0	n.s	36	Gum Arabic Tween 80 Olive oil	n.s	Chin John Hun *et al.*, 2003
Burkholderia cepacia	7.0	37	24	coconut oil, glucose	NH_4Cl	Pooja Rathi *et al.*, 2001

2.3 Purification of Lipases

Most of the commercial applications of enzymes do not always need homogeneous preparation of the enzyme. However, a certain degree of purity is required, depending upon the final application, in industries such as fine chemicals, pharmaceuticals and cosmetics. Further purification of the enzyme is a must for understanding the 3-D structure and the structure–function relationships of proteins (Aires-Barros *et al.*, 1994; Saxena *et al.*, 2003). For industrial purposes, the purification strategies employed should be inexpensive, rapid, high-yielding and amenable to large-scale operations. They should have the potential for continuous product recovery, with a relatively high capacity and selectivity for the desired product. Various purification strategies used for lipases have been reviewed several times (Palekar *et al.*, 2000; Saxena *et al.*, 2003), highlighting clearly the importance of designing optimal purification schemes for various microbial lipases. The extent of purification varies with the order of the purification steps; and this aspect has been evaluated through different purification protocols pursued by various investigators.

Purification steps involve concentration of the culture supernatant containing the enzyme by ultrafiltration, ammonium sulfate precipitation or extraction with organic solvents. Precipitation often gives a high average yield (Aires-Barros *et al.*, 1994) although with limited purification enzyme preparations are apt for use in detergent formulations. However, for certain applications, such as synthetic reactions in pharmaceutical industry, further purification is needed. Since lipases are known to be hydrophobic in nature, having large hydrophobic surfaces around the active site, the purification of lipases may best be achieved by opting for affinity chromatography,

such as hydrophobic interaction chromatography. The use of hydrophobic interaction chromatography has increased tremendously in the past few years (Imamura and Kitaura., 2000). 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 (Yadav *et al*, 1998) and two-phase systems, membrane processes, and immunopurification (Sztajer *et al*, 1989) are being used for purification of lipases. Affinity methods can be applied at an early stage, but as the hydrophobic matrices are expensive, alternatively ion exchange and gel filtration are usually preferred after the precipitation step (Litthauer *et al.* 2002; Snellman *et al.* 2002; Abdou 2003). Table2.3 summerises some of the purification strategies adopted for lipases.

Table: 2.3 strategies adopted for Purification of lipases from bacteria

Bacterium	Purification technique	Fold increase/yield	Reference
Acinetobacter spp A. calcoaceticus AAC323-1	Triton X-114-based aqueous two-phase partition	68-fold/81%	Bompensieri et al., 1996
A. calcoaceticus LP009	Ultrafiltration, gel filtration on Sephadex G-100	n.s.	Pratuangdejkul and Dharmsthiti ., 2000
A. radioresistens CMC-1	Ammonium sulfate, PD-10 column, Mono Q, phenyl-Sepharose CL-4B column chromatography	64-fold/13%	Hong and Chang., 1998
Acinetobacter sp. RAG-1	Mono Q, butyl Sepharose column, elution with Triton-X 100	10-fold/22%	Snellman *et al.*, 2002
Bacillus spp Bacillus sp.	Ammonium sulfate, acrinol treatment, DEAE-Sephadex A-50, Toyopearl HW-55F, butyl Toyopearl 650 M	7,760-fold/10%	Sugihara *et al.*, 1991; Palekar *et al.*, 2000
Bacillus sp.	Ammonium sulfate, phenyl Sepharose column	175-fold/15.6%	Nawani and Kaur., 2000
Bacillus sp.	Acetone fractionation, two acetone precipitations, octyl-Sepharose CL-4B, Q-Sepharose, Sepharose-12	3,028-fold/20%	Imamura and Kitaura ., 2000
Bacillus sp. strain 398	Ammonium sulfate, DEAE-Sepharose, butyl Toyopearl, DEAE-Sepharose	10,300-fold/30%	Kim *et al.*, 1994

Bacillus sp. THL027	Ultrafiltration, Sephadex G-100	2.6-fold/n.s.	Dharmsthiti and Luchai ., 1999
B. alcalophilus	50% ammonium sulfate, Sephadex G-100	111-fold/5%	Ghanem *et al.*, 2000
B. pumilus	Ammonium sulfate fractionation, gel filtration on Sephadex G-100	75-fold/n.s.	Jose and Kurup., 1999
B. stearothermophilus (recombinant lipase)	CM-Sepharose, DEAE Sepharose	11.6-fold/62.2%	Kim *et al.*, 2000
B. thermocatenulatus	Calcium soap, hexane extraction, methanol precipitation, Q-Sepharose (ion exchange)	67-fold/11%	Schmidt-Dannert *et al.*, 1994
B. thermocatenulatus (recombinant lipase)	Cell breakage with heat precipitation, S-Sepharose, Q-Sepharose, phenyl-Sepharose	329-fold/49%	Schmidt-Dannert *et al.*, 1996
Chromobacterium spp C. viscosum	Alginate (macroaffinity ligand), elution by NaCl, 0.5 K	1.76-fold/ 87%	Sharma and Gupta., 2001
C. viscosum Lipase A	AOT-isooctane reverse micelle system	4.3-fold/91%	Vicente *et al.*, 1990
C. viscosum Lipase B	AOT-isooctane reverse micelle system, back-extraction from micellar phase by 2.5% ethanol at pH 9.0	3.7-fold/75%	Vicente *et al.*, 1990
Pseudomonas spp Pseudomonas sp. G6	Silicone 21 defoamer, ammonium sulfate (60% saturation) fractionation	n.s./ 83%	Kanwar *et al.*, 2002

Pseudomonas sp.	Extraction, Bio-gel P-10 chromatography, Superose 12B chromatography	37-fold / 64.3%	Dong *et al.*, 1999
Pseudomonas sp. KWI-56	Acetone precipitation, gel filtration by HPLC	14-fold / 4%	Iizumi *et al.*, 1990
Pseudomonas sp. ATCC 21808	Q-Sepharose, octyl-Sepharose, elution with isopropanol	159-fold/56%	Kordel *et al.*, 1991
Pseudomonas sp. Yo103	Ammonium sulfate precipitation, DEAE-cellulose, Sephadex G-200	62-fold/3.7%	Kim *et al.*, 1997
P. aeruginosa	Ammonium sulfate precipitation, hydroxyapatite column chromatography	518-fold/n.s.	Sharon *et al.*, 1998
P. aeruginosa EF2	Ultrafiltration, anion-exchange chromatography (Mono-Q), FPLC gel filtration (Superose)	31-fold/18%	Palekar *et al.*, 2000

2.4 Lipase Assay

One important aspect of lipolytic enzymes is the unique physico-chemical character of the reactions they catalyse at lipid-water interfaces, involving interfacial adsorption and subsequent catalysis. Most of the lipases are water-soluble enzymes acting on water-insoluble substrates (supersubstrates). The heterogeneous character of this catalysis makes it difficult to accurately quantitate both the amount of interface (specific surface) (Frédéric *et al.*, 2000) as well as the interfacial parameters (such as the interfacial tension, surface viscosity, surface potential, etc.) responsible for the "interfacial quality" of the substrate (Panaitov *et al.*, 2000), on which the lipolytic process greatly depends. The emulsification of the water-insoluble substrates, which requires the presence at the interface of

surface active amphiphiles such as detergents, other lipids, proteins, etc., can therefore drastically influence lipase activity measurements: non-specific inhibition of lipases by proteins present at the oil/water interface is one well-known phenomenon of this kind (Gargouri *et al.*, 1986).

Lipases are often analyzed by their hydrolytic action on triglycerides in a heterogeneous reaction medium of water and oil. As the medium is heterogeneous, the addition of any amphiphilic compound to the system will have effects on the interface and often on the enzyme itself. Some lipases, such as gastric lipases, rapidly become denatured at an interface with pure tributyrin emulsion, and it is therefore impossible to assess the interfacial activation with substrates of this kind. Esters which are partly soluble in water may form monomolecular adsorption films on the surface of the air bubbles which are produced upon stirring the reaction mixture. This phenomenon is responsible for a great disparity between initial velocity measurements, depending on whether or not mechanical stirring methods are used. It has in fact been established that the quality of the lipid/water terms of the orientation and conformation of the film-forming molecules, the molecular and charge surface:

Table: 2.4 Analytical methods for lipases (Schmid, *et al.*, 1998)

Method	Principle	Advantages	Disadvantages
pH stat potentiometric	Determination of fatty acids liberated upon hydrolysis	• Continuous kinetic methods for the determination of initial rates	• Expensive equipment • Added emulsifiers often modify reaction • Not applicable under acidic pH conditions conditions
Back titration	Titration of liberated fatty acids after defined exposure to lipase action	• Simple	• No continuous kinetic data
Colorimetry	Liberation of a reporter group from a synthetic ester (e.g. p-nitrophenyl palmitate or dilauryl glycerol resorufin ester)	• Fast and automatic kinetic measurements	• Unnatural substrates
Determining the hydrolysis of lipid films spread at the air/water interface	Hydrolysis of a lipid monolayer at constant surface pressure with continuous supply of substrate (zero order trough)	• Kinetic measurements in the absence of added emulsifier and under controlled "interfacial quality"	• Expensive equipment • Time-consuming • Trained experimentalist necessary

densities, the interfacial water structure, the surface viscosity are the most decisive parameters when working with lipolytic enzymes (Verger *et al.*, 1997) Commonly used lipase assays are documented in the table 2.4.

2.5 Properties of bacterial lipases

Lipases from several microorganisms have been studied extensively and, based on their properties, used in various industries. Various properties of bacterial lipases viz. molecular weight, pH and temperature optima, stability, and substrate specificity reported in the literature are summarized in the in the Table:

2.5.1 Stability in organic solvents

Stability in organic solvents is desirable in synthesis reactions. From the available literature, it can be inferred that lipases are generally stable in organic solvents, with few exceptions of stimulation or inhibition. Acetone, ethanol and methanol enhanced the lipase activity of *B. thermocatenulatus* (Schmidt *et al.*, 1994), whereas acetone was inhibitory for *P. aeruginosa* YS-7 lipase and hexane for *Bacillus* sp. lipase (Sugihara *et al.*, 1991). Lipase from *A. calcoaceticus* LP009 was highly unstable with various organic solvents (Dharmsthiti *et al.*, 1998).

2.5.2 Effect of metal ions

Divalent cations such as calcium often stimulate enzyme activity. This has been suggested to be due to the formation of the calcium salts of long-chain fatty acids (Godtfredsen., 1990). Calcium-stimulated lipases have been reported in the case of *B. subtilis* 168 (Lesuisse *et al.* 1993), *B. thermoleovorans* ID-1 (Lee *et al.* 1999), *P. aeruginosa* EF2 (Gilbert *et al.*1991), *S. hyicus* (Van Oort *et al.*, 1989), and *Acinetobacter sp.* RAG-1 (Snellman *et al.* 2002). Further, lipase activity is in general inhibited drastically by heavy metals like Co2+, Ni2+, Hg2+and Sn2+and slightly inhibited by Zn2+ and

Mg2+ (Patkar and Bjorkling 1994).However, the lipase from *A. calcoaceticus* LP009 was stimulated by the presence of Fe3+ and its activity was reduced by less than 20% on addition of various other ions (Dharmsthiti *et al.* 1998).

2.5.3 Lipase inhibitors

Lipase inhibitors have been used in the study of structural and mechanistic properties of lipases. Further, the search for lipase inhibitors is also of pharmacological interest. Lipase inhibitors are used for designing drugs for the treatment of obesity and the problem of acne.

Compounds that do not act directly at the active site, but inhibit lipase activity by changing the conformation of lipase or interfacial properties are defined as non-specific inhibitors. Surfactants (Iizumi *et al.*, 1990; Patkar and Bjorkling., 1994) and bile salts (Wang *et al.*, 1999) belong to this group of inhibitors are found to have a positive effect on lipase activity.

Specific inhibitors are those compounds, which directly interact with the active site of the enzyme. Such inhibitors can be either reversible or irreversible. Specific reversible inhibitors include: (1) boronic acid derivatives, which form reversible but long-lived complexes with the active site serine of lipases (Lolis and Petsko., 1990) and (2) substrate analogues including triacylglyceride analogue glycerol triether, which is also a competitive inhibitor of pancreatic lipase (Lengsfeld and Wolfer., 1988).

Lipases belong to the class of serine hydrolases with the catalytic triad as Ser-His-Asp/Glu. Therefore, serine inhibitors are potential irreversible active-site lipase inhibitors, e.g. phenylmethylsulfonyl fluoride (PMSF), phenylboronic acid, diethylp-nitrophenyl phosphate. In contrast, the lipase from *A. calcoaceticus* LP009 was

not inhibited by PMSF (Dharmsthiti *et al.* 1998). Generally, lipases are not sulphydryl proteins; and thus in most lipases neither free –SH nor S–S bridges are important for their catalytic activity. This is substantiated by the use of 2-mercaptoethanol.p-chloromercuric benzoate and iodoacetate, which have no detectable effect on lipase from *A. calcoaceticus* LP009 (Dharmsthiti *et al.*, 1998). Further, EDTA does not affect the activity of most lipases (Sugihara *et al.*, 1991; Kojima *et al.*, 1994). However, it is inhibitory to lipases from *Bacillus sp.* THL027 (Dharmsthiti and Luchai., 1999) and *A. calcoaceticus* LP009 (Dharmsthiti *et al.*, 1998). Tryptophan residues play an important role in maintaining the conformation of lipases (Patkar and Bjorkling., 1994). Modification of tryptophan residues in lipases from P. fragi CRDA 037 (Schuepp *et al.*, 1997) by N-bromosuccinimide leads to decreased lipase activity.

2.5.4 Substrate specificity

Microbial lipases may be divided into three categories: namely nonspecific, regiospecific and fatty acid-specific, based on the substrate specificity. Nonspecific lipases act at random on the triacylglyceride molecule and result in the complete breakdown of triacylglyceride to fatty acid and glycerol. Examples of this group of lipases include those from *S. aureus, S. hyicus* (Jaeger *et al.*, 1994), and *Chromobacterium viscosum* (Jaeger *et al.* 1994). In contrast, regiospecific lipases are 1,3-specific lipases which hydrolyze only primary ester bonds (i.e. ester bonds at atoms C1 and C3 of glycerol) and thus hydrolyze triacylglyceride to give free fatty acids, 1,2(2,3)-diacylglyceride and 2-monoacylglyceride. Extracellular bacterial lipases are regiospecific, e.g. those from *Bacillus sp.*(Sugihara *et al.*, 1991; Lanser *et al.*, 2002), *B. subtilis* 168 (Lesuisse *et al.*, 1993), *Bacillus sp.* THL027 (Dharmsthiti and Luchai., 1999). The third

group comprises fatty acid-specific lipases, which exhibit a pronounced fatty acid preference. *Achromobacterium lipolyticum* is the only known bacterial source of a lipase showing fatty acid specificity (Davranov., 1994). However, lipases from *Bacillus sp.* (Wang et al.1995) show specificity for triacylglycerides with long-chain fatty acids, while lipases from *B. subtilis* 168 (Lesuisse et al., 1993), *Bacillus sp.*THL027 (Dharmsthiti and Luchai., 1999), prefer small or medium-chain fatty acids.

Another important property of lipases is their enantio-stereoselective nature, wherein they possess the ability to discriminate between the enantiomers of a racemic pair. Mostly lipases from Pseudomonas family fall in this category (Reetz and Jaeger 1998). The stereospecificity of a lipase depends largely on the structure of the substrate, interactions at the active site and the reaction conditions (Muralidhar et al. 2002). The lipase from *P. cepacia* is a popular catalyst in organic synthesis (Kazlauskas and Bornscheuer 1998) for the kinetic resolution of racemic mixtures of secondary alcohols in hydrolysis, esterification and transesterification (Schulz *et al.*,2000).

2.6 Biotechnological application of lipase

Lipases find their way into industries based on their specificities towards various triglycerides. Since this enzyme acts at the oil–water interface, it can be used as a catalyst for the preparation of industrially important compounds (Woolley *et al*,1994) especially in the area if organic synthesis.

Lipases 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. As lipases act on ester bonds, they have been used in fat splitting, interesterification (transesterification), development of different flavours in cheese, improving pallatability of beef fat for making dog food, etc. A current application involves using lipases in water-deficient organic solvents for synthesizing different value-added esters from organic acids and alcohols. Lipases which are stable and work at alkaline pH, say 8 to 11, which are usually the suitable wash conditions for enzymated-detergent powders and liquids, have also been found, and these hold good potential for use in the detergent industry (Langrand *et al* , 1990) . 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.

In 1995, two bacterial lipases were introduced 'Lumafast' from Pseudomonas mendocina and 'Lipomax' from P. alcaligenes by Genencor International (Jaeger and Reetz., 1998). Gerritse *et al.* (1998) reported an alkaline lipase, produced by *P. alcaligenes* M-1,

which was well suited to removing fatty stains under conditions of a modern machine wash.

2.6.1 Applications in Food and Dairy Industries:

Lipases are extensively used in the dairy industry for the hydrolysis of milk fat. They are used as a flavour enhancing agent during cheese ripening, manufacture of cheese like products and the lipolysis of butter fat (Falch *et al.*, 1991). Lipases release short chain fatty acids (C4-C6) that imparts a sharp and tangy flavour to the cheese. Where as the release of the medium chain fatty acids (C12-C14) helps in the development of soapy flavour to the milk products. (Falch *et al.*, 1991). These enzymes are commonly used in the production of a variety of products ranging from fruit juices, baked foods and vegetable fermentation to dairy enrichment. (Zalacain *et al.*, 1994). Lipases especially of microbial origin have been used for the production of desirable flavours in cheese and other foods and for the interesterification of fats and oil to produce modified acyl glycerols, which cannot be obtained by convential chemical interesterification. During storage, one of the most important changes that occur in the lipid fraction is the hydrolysis of triacylglycerols, catalysed by lipase retaining non-esterified fatty acids, which are very important for the characteristic flavour of these products (Heidt *et al.*, 1994).

Buisman *et al.* (1998) used immobilized lipases from *C. antarctica* (CAL-B), *C. cylindracea AY*30, *Humicola lanuginosa*, *Pseudomonas* sp. and *Geotrichum candidum* for the esterification of functionalized phenols for synthesis of lipophilic antioxidants to be used in sunflower oil. Cao *et al.* (1997) reported a lipase-catalysed solid-phase synthesis of sugar fatty acid esters.

Alcoholises of cod liver oil for the production of Omega-3 polyunsaturated fatty acids are investigated by using Pseudomonas lipase (Zuyi *et al*, 1993). Ester exchange for vegetable oil modification involving microbial lipases suggested that lipases converted triolein in vegetable oil to a monoleate and it could be used for improving inexpensive vegetable oil with high industrial qualities. Unilever obtained a series of patents for the interesterification of fats and acylglycerols (Unilever., 1986).

Proteases and lipases from other sources have increasingly been used in cheese making. Depending on the chain-length specificity of a given lipase, its addition to a milk product may enhance the flavor of the cheese, accelerate the cheese ripening, or assist in the preparation of ™enzymemodified cheeses° (EMC), an important commercial flavour used in the USA for the manufacture of dips, sauces, dressings, crackers, etc. EMC is produced from cheese curd by the addition of lipases at elevated temperatures, increasing the content of free fatty acids about tenfold.

2.6.2 Lipase in detergent industry:

Detergent industry is the prime field which consumes the largest share of the microbial lipase produced. The addition of lipases to detergent formulations has been investigated in the context of removal of fat stains (Wolff *et al*, 1997) and in situ generation of peracid bleach by perhydrolysis (Kirk *et al* , 1994).

After the great commercial success of proteases as detergent additives, the enzyme industry undertook major efforts to introduce lipases as a second group of detergent enzymes. It was hoped that lipases could compete with chemical surfactants in the detergent formulation, and thus respond to changing detergent formula in view

of lower wash temperatures and ecologically benign components. Standard wash liquids contain anionic and nonionic surfactants, oxidants, and complexing agents at a pH of about 10 and temperatures around 50°C, which is a rather hostile environment for enzymes. It later turned out, however, that suitable lipases can prevent redeposition of fat material on washed textiles, thus exerting a secondary effect on laundry cleaning.

Lipases are generally added to the detergents primarily in combination with proteases and cellulases. At present they are in extensive use in household detergents and industrial cleaners, and in leather processing (Misset *et al*, 1993). Lipases have also been used in the formulations prepared to clean drains clogged with food and or non-food plant-material containing deposit (Genex, 1986). Here they are used in association with pectinase. To be a suitable additive in detergents, lipases should be both thermophilic and alkalophilic and capable of functioning in the presence of the various components of washing powder formulations (Jaeger *et al.*, 1994).

Misset *et al.* (1994) tailored *Pseudomonas alkaligenes* lipase for elevated activity at washing conditions, such as alkaline pH high temperature (up to 60 °C) and the presence of non-ionic and anionic detergents. This enzyme was also found to be stable during storage in the surfactants and in the washing machine. Thermophilic lipases have been reported from many bacterial species (Jaeger *et al.*, 1994; Ushio, *et al.*, 1996). The stability and activity of detergent enzymes were crucial factors, which were greatly improved by the so-called 'surfactant lipases' (Christensen *et al* ., 1990). Lipolase, which was introduced by Novo, was a pioneer in this regard. Unilever, Cosmo Oil and Procter & Gamble are major companies working on

recombinant DNA technology for surfactant lipases (Jaeger *et al*, 1994).

2.6.3 Lipase in the leather industry:

Leather processing involves the removal of subcutaneous fat, dehairing and stuffing. An enzyme preparation that contained lipases in combination with other hydrolytic enzymes such as proteases would open a new avenue in leather processing. A new enzymic process for the production of hides and skins, ready for tanning, involved steps of soaking, washing, dehairing and bathing in aqueous baths, where each bath had a pH of 8–13 and contained an alkalophilic lipase (Macrae *et al* .,1985). Tanning processes are usually performed in an alkaline environment, so alkalophilic microbes ought to be better for exploration. Many *Bacillus* sp. strains, which grew successfully under highly alkaline conditions, were found to be useful in leather processing (Haalck *et al*, 1992).

2.6.4 Biosurfactants:

Surfactants are amphiphilic molecules which are widely used in many industries. Sugar fatty acid esters are in the group of non-ionic surfactants and have high emulsifying, stabilizing, detergency and other useful effects. They are utilizable in pharmaceutical, cosmetic, food industries and so forth. Glucose sterate could be used as bread softening agent, sorbitol and sugar monoesters of lauric and steric acid possess antitumor and growth inhibiting activity. Lipase may also be useful in the synthesis of whole range of amphoteric biodegradable surfactants namely aminoacid based esters and amides. Sugar fatty acids are produces from renewable inexpensive and readily available substances. They are harmless to the environment due to their complete biodegradability under aerobic and anaerobic

conditions. They are non-toxic, non- skin irritants, odourless, and tasteless (Acros *et al.*, 1997).

2.6.5 Medical and Pharmaceuticals

Lipases are used for the resolution of recemic alcohols in the preparation of some prostaglandins, steroids and carboxylic nucleosides analogues. They are also used in the regioselective modification of polyfunctional organic compounds which have a great significance in the treatment of AIDS (Margolin *et al*, 1989). Another impressive example of the commercial application of lipase in the resolution of recemic mixtures in the hydrolysis of epoxy ester alcohols.The reaction products, (R) –Glycidyl esters and (R) – Glycidol are readily converted to (R) – and (S) – Glycidyltosylates which are attractive intermediates for the preparation of optically active beta blockers and wide range of other products. A similar technology has been commercialised to produce 2(R), 3(S) - Methylmethoxy phenyl glycidate, the key intermediate in the manufacture of optically pure cardiovascular Diltiazem (Bronemann *et al.*, 1989).

Enantioselective interesterification and transesterification have great significance in pharmaceuticals for selective acylation and deacylation. Chillemi *et al* (1998) performed the chemoenzymic synthesis of lysophosphatidylnucleosides, which involved lipase-catalysed transacylation. The synthesis was also applied to the preparation of *O*-(1-*O*palmitoyl-*sn*-glycero-3-phosphoryl) conjugates of Acyclovir and AZT, which are of potential pharmacological interest. A method was developed by Jimenez *et al.* (1997) to synthesize methyl (*R*)- and (*S*)-2-tetradecyloxiranecarboxylate

through sequential kinetic resolution catalysed by lipases. Both the enantiomers are a potent anti-diabetic and antioxidant agent.

Goto *et al.* (1996) also studied the enzymic resolution of racemic ibuprofen by surfactant coated lipases in organic media. A new method was developed for preparing triacylglycerols containing highly unsaturated fatty acids in large amounts, which involved treating one or a mixture of monoacylglycerols or diacylglycerols containing highly unsaturated fatty acids (e.g. docosahexaenoic acid) with one or more lipases. The product has improved learning functions, activates the immune system and has antiarteriosclerotic, anti-tumor and anti-allergic activities.

2.6.6 Biosensors:

Sensing lipids and lipid-binding proteins are a developing technology (Spener *et al*, 1992). In the fat and oil industry, in food technology and in clinical diagnosis, the quantitative determination of triacylglycerols is of great importance (Schoemaker *et al* , 1991). Chemical methods for the analysis are rather costly and time-consuming. A promising new method involves the manipulation of microbial lipases as a biosensor.

Shoemaker et al.(1991) developed a promising method, which involved the use of microbial lipase, in which the glycerol liberated during lipid hydrolysis was oxidized by glycerol dehydrogenase. NADH formed during the reaction was measured by fluorescence spectroscopy.

2.6.7 Lipase in pesticides:

Fine and intermediate chemicals makers emphasize new products and processes for the pesticide industry via lipases, in view of its potential for decreasing costs and environmental contamination (Pandey, 1993).A variety of pesticides (insecticides, herbicides, fungicides or their precursors) made with the application of lipases are currently in use (Sugai et al , 1995., Bianchi et al, 1992., Pan et al, 1990). The most important application of lipases has been in the organic synthesis of pesticides for the production of optically active compound (Reddy *et al* , 1992). Akita et al.(1995) described a highly stereospecific synthesis of the versatile chiral synthon possessing two stereogenic centres, which was subsequently converted into a homochiral intermediate for the synthesis of the biologically active potent pesticide nikkomycin-B.

In another patent, the synthesis of a new ivermectin fatty acid ester derivative esterified by lipase has been reported. Apart from its usefulness in human and veterinary medicines, ivermectin was found to be active against many pests (Merck, 1990).

2.6.8 Lipases in Environmental management:

Bioremediation for waste disposal is a new avenue in lipase biotechnology. Oil spills during rigging and refining, oil-wet night soils and shore sand, and lipid-tinged wastes in lipid processing factories and restaurants could be well handled by the use of lipases of different origins (Benjamin, 1997., Krempen et al, 1995). Lipases have been used extensively in wastewater treatment (Kurita-Water, 1994). Dauber and Boehnke (1993) devised a technology to convert dewatered sludge in the factories to biogas, in which an enzyme mixture including lipase was used. Another important application of lipases has been reported in the degradation of polyester waste into

useful products, especially for the production of non-esterified fatty acids and lactones (Piras et al,1994., Nishida et al, 1994). The broadening use of lipases in bioremediation has achieved more importance with its successful application to the removal of biofilm deposits from cooling water systems (Nisshin Oilmills, 1993), to the manufacture of liquid soap, to the upgrading of waste fat

2.6.9 Biopolymers:

The sterioselectivity of lipase is useful in the synthesis of optically active polymers. These polymers are asymmetric reagents and are used as absorbents. In the field of liquid crystals, suitable monomers can be prepared by lipase catalysed transesterification of alcohols. (Pavele 1992) The use of chiral Glycidyltosylates for the prepration of ferroelectric liquid crystals have also been reported. (Margolin *et al*, 1990).

2.6.10 Lipases in Cosmetic and Perfume industry:

The overwhelming interest of technocrats in screening lipases for use in the cosmetic and perfume industry has mainly been due to its activity in surfactants and in aroma production, which are the main ingredients .in cosmetics and perfumes (Kao, 1995). Monoacylglycerols and diacylglycerols, prepared by the lipase-catalysed esterification of glycerol, are useful as surfactants in cosmetics(McCrae *et al.*, 1990). Izumi et al.(1997) performed the transesterification of 3, 7-dimethyl-4, 7-octadien-1-ol with lipases from various microbial sources to prepare rose oxide, which is an important fragrance ingredient in the perfume industry. Miyamoto *et al.*(1995) obtained a patent for the utilization of lipases in the production of glycerine mixtures. This was to be used as an external

preparation or by application directly to skin. Another patent involved lipases as the main catalyst for cosmetic production from plant ash. This was claimed to be an efficient skin-conditioning agent. Unichem International has recently launched the production of isopropyl myristate , isopropyl palmitate and 2- ethlhexyl palmitate (Young *et al.,* 1990)for use as a emollient in personal care products like skin and sun-tan creams and bath oils.

2.6.11 Lipases in pulp and paper industry

'Pitch,' or the hydrophobic components of wood (mainly triglycerides and waxes), causes severe problems in pulp and paper manufacture (Jaeger and Reetz, 1998). Lipases are used to remove the pitch from the pulp produced for paper making. Nippon Paper Industries, Japan, have developed a pitch control method that uses the Candida rugosa fungal lipase to hydrolyze up to 90% of the wood triglycerides.

2.6.12 Lipases in organic synthesis

Use of lipases in organic chemical synthesis is becoming increasingly important. Lipases are used to catalyze a wide variety of chemo-, regio-, and stereoselective transformations (Berglund and Hutt, 2000).Majority of lipases used as catalysts in organic chemistry are of microbial origin. These enzymes work at hydrophilic–lipophilic interface and tolerate organic solvents in the reaction mixtures. Use of lipases in the synthesis of enantiopure compounds has been discussed by Berglund and Hutt (2000).The enzymes catalyze the hydrolysis of water-immiscible triglycerides at water–liquid interface. Under given conditions, the amount of water in the reaction mixture will determine the direction of lipase-catalyzed reaction.

2.6.13 Lipases in ester synthesis

Lipases have been successfully used as catalyst for synthesis of esters. The esters produced from short-chain fatty acids have applications as flavoring agents in food industry (Vulfson, 1994). Methyl and ethyl esters of long-chain acids have been used to enrich diesel fuels (Vulfson, 1994). Esterification of five positional isomers of acetylenic fatty acids (different chain lengths) with n-butanol was studied by Lie *et al.* (1998), using eight different lipases

2.6.14 Lipases in oleochemical industry

Use of lipases in oleochemical processing saves energy and minimizes thermal degradation during alcoholysis, acidolysis, hydrolysis, and glycerolysis (Vulfson, 1994; Bornscheuer, 2000). Although lipases are designed by nature for the hydrolytic cleavage of the ester bonds of triacylglycerol, lipases can catalyze the reverse reaction (ester synthesis) in a low water environment. Hydrolysis and esterification can occur simultaneously in a process known as interesterification. Depending on the substrates, lipases can catalyze acidolysis (where an acyl moiety is displaced between an acyl glycerol and a carboxylic acid), alcoholysis (where an acyl moiety is displaced between an acyl glycerol and an alcohol), and transesterification (where two acyl moieties are exchanged between two acylglycerols)(Balcao *et al.*, 1996).

2.7 Bacillus Lipase

Bacillus species of industrial importance are vastly applied in the production of several biological products (Schmidt, 2004). These species are important organisms for both fundamental research and industrial applications. Bacilli currently account for 60% of the commercially available proteins synthesized on an economical scale (Bron *et al.*, 2004). Majority of these proteins are homologous proteins that are naturally secreted into the growth medium, such as alkaline proteases and amylases (Quax, 2003). Certain species from the genus are applied in the development of expression systems for recombinant protein production (Schallmey *et al.*, 2004). The demand for expression systems capable of overexpresing both homologous and heterologous proteins is rapidly increasing (Hazawa-Cho, 1999).However, some *Bacillus* species have remarkable industrial applications (Harwood, 1992) e.g. *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* have a long history of safe commercial application in the food, detergents and pharmaceuticals industries (Harwood, 1992; Priest, 1993; Bron *et al.*, 1999).

Bacilli are well-known high-level producers of a variety of extracellular proteins (Yasuhiko *et al.*, 2000). Their capacity to produce and secrete large quantities (20-25 g/l) of extracellular proteins has placed them amongst the most important industrial protein producers (Schallmey *et al.*, 2004). These organisms continue to be dominant bacterial workhorses in microbial fermentations and have been extensively applied in the production of useful biochemicals, antibiotics, insecticides and industrial enzymes.

Bacillus species have been reported earlier producing lipolytic enzymes under alkaline conditions (Lindsay *et al*, 2000). Lipases

from *Bacillus subtilis* and *B. pumilis* (Moller, *et al*, 1991) reported earlier showed optimal activity and stability at extreme alkaline pH values 9.5 (Nthangeni, 2001). A recombinant *B. licheniformis* lipase showed maximum activity at pH 10–11.5 (Nthangeni, 2001). There are thermotolerant lipases isolated from *B. thermoacetenulatus* (Lee, 1999) and *B. thermoleovorans*(Rua, 1997) which displayed maximum activity and stability at moderate alkaline pH values. Six extracellular lipases and two esterases have been described so far to occur in *Bacillus* species (Dartois *et al*, 1994., Moller *et al*, 1991., Alvarez-Macarie *et al*, 1999., Eggert *et al*, 2000., Meghji *et al*, 1990). Table 2.6 summarizes some of the lipases reported form Bacillus sp.

Lipases are today an attractive area of research both at national and global front because of their distinguished catalitical properties compared to other hydrolases. But it is interesting to note that though there are immense reports of bacterial lipases isolated from terrestrial environment, there are no significant reports of a bacterial lipase being isolated and characterized for industrial applications from a marine environment.

Table: 2.6 Biochemical properties of extracellular lipases and esterases from *Bacillus* species (Eggert *et al.*, 2002)

Source of lipolytic Enzyme	Mol. mass (kDa)	pH (Opt.m)	Temp (Optm)°C	Temp (Stab)°C	Substrate specificity	Conserved peptide sequence	Comments	Reference
B. subtilis 168 LipA -	19.3	10	35–40	45	pNP-C8,C14 TG-C8	A–H–S–M–G	Tendency to form aggregates	Eggert *et al.*, 2000
LipB	19.5	10	35–40	45	pNP- C8,C14	A–H–S–M–G	Tendency to form	Eggert *et al.*, 2000
B. subtilis NRRL 365 Esterase I	365	8.0	n.d	. n.d	pNP-C2-C3	n.d.		Meghji *et al.*, 1990
Esterase II	. 105.0	8.0	n.d.	. n.d	pNP-C2	Heterodimer subunits with Mr 48 kDa and Mr 57 kDa	n.d.	Meghji *et al.*, 1990
B. pumilus	19.3	30	40	n.d	n.d	. A–H–S–M–G	80.1and 77.3% similarity to *B. subtilis* LipA and LipB, respectivly	Mo ller *et al.*, 1991

B. thermocatenulatus BLT1	16.0	7–8	60–70	40	pNP-C10 12	n.d.	Tendency to form aggregates (_750 kDa	Schmidt-Dannert, *et al.*, 1997
BLT2	43.0	8–9	60–70	50	pNP-C10 TG-C4	A–H–S–Q–G	Tendency to form aggregates (_500 kDa)	Schmidt-Dannert, *et al.*, 1997
B. stearothermophilus L1	43.0	9–10	60–65	55		A–H–S–Q–G	94.2% similarity to *B. thermocatenulatus* BLT2	Kim *et al.*, 2000
B. thermoleo_orans ID-1	43.0	7–8	70–75	50	pNP-C8 TG-C3,C12	A–H–S–Q–G	96.1% similarity to *B. pumilus* lipase	Cho *et al.*, 2000
B. licheniformis Lipase	19.2	n.d.	n.d.	n.d.	pNP-C6 TG-C8	A–H–S–M–G	95.4% similarity to *B. thermocatenulatus* BLT2	Accession no AJ297356
Esterase	81.3	8–8.5	55	50	n.d.	A–H–S–M–G	Forms trimers of Mr 95 kDa	Alvarez-Macarie *et al.*, 2000
B. circulans	30.0	8.5–9.5	60	70	pNP-C6-C8	n.d.	Very low activity (4.9 Umg−1 towards TG-C8)	Kademi *et al.*, 2000a, 2000b
B. spec. THL027	69.0	7	70	n.d.	pNP-C3-C4 TG-C8	n.d.	tendency to form aggregates	Dharmsthiti, et al.1999

Chapter 3

MATERIALS AND METHODS

3.1 MICROORGANISMS

Bacteria isolated form the sediment, and sea water of the coastal areas of Tiruvananthapuram, Rameshwaram and Cochin, and available as stock cultures in the Microbial Technology Laboratory, Department of Biotechnology, CUSAT, Cochin were used for the study. Further isolates obtained from the sediment and seawater of coastal belts of other parts of South India were also used for the study. The cultures were maintained in ZoBell's marine agar at 4^0C and were subcultured periodically.

3.2 MEDIA

3.2.1 Marine ZoBell's medium

Marine ZoBell's medium (Hi Media) was used for the present study

3.2.2 Minimal salts media

Minimal media with the composition mentioned below was used as the basal media for evaluating the efficiency of the strains for their ability to use various lipid substrates as the sole source of carbon.

Composition

Na_2HPO_4	0.6g
KH_2PO_4	0.3 g
NaCl	0.2 g
NH_4Cl	0.1g
$MgSO_4.7H_2O$ (1M)	0.200 ml
$CaCl_2.2H_2O$ (0.1M)	0.100 ml
Distilled water	100 ml
pH	7.0

The various components of the media was added to distilled water taken in a 250ml conical flask, and added with the desired lipid substrate and autoclaved.

3.2.3 Tween 80 agar Plates

Composition

ZoBell's marine agar (HiMedia)

Tween 80 1.0%(v/v)

Preparation of Tween 80 agar Plates

1. 1% Tween 80 (v/v) was added to ZoBell's Marine agar and sterilized.
2. The media was poured into sterilized plates.
3. Cultures were spot inoculated to the cooled plates and incubated for 24-48 hrs at room temperature.

4. Plates were checked for precipitated zones around the colonies for the detection of lipase producing strains.

3.2.4 Tributyrine Agar Plates

Composition

ZoBell's marine agar (HiMedia)

Tributyrine	1.0% (v/v)

Preparation of Tributyrine agar plates.

1. 1% Tributyrine (v/v) was added to ZoBell's Marine agar and sterilized.
2. The media was poured into sterilized plates.
3. Cultures were spot inoculated to the cooled plates and incubated for 24-48 hrs at room temperature.
4. Plates were checked for clearing zones around the colonies for the detection of lipase producing strains.

3.2.5 Olive Oil -Rhodamine B plates

Composition

Olive oil	3 ml
Agar	2 g
Rhodamine B	1 g
50mM Tris HCl buffer containing1 mM $CaCl_2$	100 ml

Preparation of Rhodamine B plates with olive oil.

1. Hundred ml of 50 mM Tris HCl Buffer containing 1 mM $CaCl_2$ added with 2g agar taken in a conical flask was autoclaved
2. The olive oil was sterilized separately in a hot air oven at 170° C for 2 hrs.
3. Rhodamine B was filter sterilized.
4. The above constituents were mixed properly with the help of a magnetic stirrer under aseptic conditions.
5. The media was poured to the sterilized plates under aseptic conditions and was allowed to cool to room temperature.
6. Punched wells were made in agar plates using sterile punching syringe.
7. The culture supernatants were added to the wells.
8. The plates were incubated at 37° C for 24-48 hrs.
9. The plates were observed in the UV light for the detection of potential lipase producing strains.

3.2.6 Analytical Methods.

3.2.6.1 Growth estimation

Growth was estimated in terms of cell concentration by turbidometry 600 nm. Cell dry weight was used wherever it was found necessary. This was done by centrifuging the fermentation broth at 10000 rpm and cell pellet obtained was suspended in sterile distilled water and dried at 110°C in a preweighed dry filter paper until constant weight was achieved.

3.2.6.2 Lipase Assay:

Lipase activity was estimated using a spectrophotometric assay with p-nitrophenyl esters (pNPA, pNPB, pNPC) as a substrate,

which was dissolved in acetonitrile at a concentration of 10 mM. Subsequently, ethanol and 50 mM Tris HCl buffer (pH 7.0) were added to a final composition of 1:4:95 (v/v/v) of acetonitrile/ethanol/buffer respectively (Bulow *et al*, 1987). No ethanol was added when pNPA was used as substrate. The cell free supernatant (0.2 ml) was added to the substrate solution (2.3 ml), and then the mixture was incubated at 37°C. After 30 min, enzyme activity was measured by monitoring the change in absorbance at 410 nm that represents the amount of released p-nitrophenol (PNP). The enzyme activity was calculated as µmol of p-NP released per minute per ml of enzyme solution under standard assay conditions.

3.2.6.3 Protease Enzyme Assay

Protease activity was determined by caseinolytic method of Kunitz (1947) with minor modification. In this method, the TCA soluble fractions formed by the action of protease enzyme on the protein substrate Hammerstein casein was measured by the increase in absorbance at 280 nm. The method followed is as described below.

1. Two ml of 1% (w/v) Hammerstein casein prepared in 0.05 M carbonate-bicarbonate buffer (pH 7.0) and 0.5ml of the same buffer were preincubated at 37^0C for 10 min.
2. To the above solution 0.5 ml of diluted enzyme solution was added and incubated at 40^0C for 30 min.
3. The reaction was arrested with 2.5 ml of 0.44M 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 (Kubota, Japan)
5. The absorbance of the clear supernatant was measured at 280nm in UV-Visible spectrophotometer (Shimatzu, Japan) against suitable blanks. The TCA soluble fractions of protein formed was 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 ml of the reaction mixture per minute under the assay conditions.

3.2.6.4 Protein Estimation

Protein content was determined according to the method of Lowry *et al* (1951) using Bovine Serum Albumin (BSA) as the standard.

Reagents

(a) Solution containing 2% of Sodium Carbonate in 0.1N Sodium hydroxide.
(b) 0.5% solution of Cupric Sulphate in distilled water
(c) 1% solution of Sodium Potassium tartrate in distilled water
(d) *Working reagent: To 100 ml of solution (a) add 1ml each of solution (b) and solution (c) was added
(e) *1:1 Folin and Ciocalteau's phenol reagent diluted with distilled water

*Prepared fresh before use.

Estimation

An aliquot of 0.2ml of sample was made up to 2 ml with distilled water and 5 ml freshly prepared working reagent (d) was added,

mixed thoroughly and incubated for 10 min. To this 0.5ml of solution (e) was added and incubated for 30 min followed by measuring the absorbance at 750nm in a UV-Visible spectrophotometer (Shimatzu, Japan).

3.2.6.5 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

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

** All the experimental data were statistically analysed using Microsoft Excel.

3.3 SCREENING OF BACTERIA FOR THE LIPASE PRODUCTION.

Screening of bacterial isolates for selection of potential lipase producing strain was carried out in three phases. First phase included primary screening of lipase production tested employing plate assay. Positive cultures were then subjected to secondary screening which was done on Rhodamine B plates. Positive cultures obtained after secondary screening were further subjected to final screening which was done using different lipid substrates. The details are given below.

3.3.1 Primary Screening

As a part of the primary screening 280 bacterial isolates were screened for lipase activity using Tween 80 agar (Sierra *et al*,1957) and Tributyrine agar (Cardenas *et al*, 2001). The substrates were incorporated in to the ZoBell's marine agar medium at 1% level (v/v) concentration. The cultures were inoculated as spot inoculum and incubated at 28±2 (RT) for 48-72 hrs. Formation of halo zones (Tributyrine Agar) or precipitates around the colony(Tween 80 Agar) were considered as positive. The positive isolates were short-listed.

3.3.2 Secondary Screening

All those isolates which showed lipase production during primary screening were further subjected to secondary screening in olive oil-Rhodamine B plates (Kouker *et al*, 1987). This is a specific plate assay for the detection of specific lipase producing strain from those of the esterase producers. Culture supernatant was added to wells punched in the plates and the plate was incubated at 37°C for 24-36 hrs. The plates were observed in the UV light for the detection of potential lipase producing strains. The positive cultures were short listed for further study.

3.3.3 Final screening

Short listed cultures obtained after secondary screening, were grown with four different lipid substrates, (Olive oil, Coconut oil, Tween 80 and Tributyrine) in ZoBell's marine broth and the minimal media and evaluated for their lipase activity. Lipid substrate was added to these media at 1% (v/v) level. The

lipolytic activity of the culture supernatant was estimated after 48 hrs of incubation.

3.3.4 Selection of potential strain

A single potential lipase producing strain was selected based on the results obtained from the final screening and was used in further studies.

3.4 IDENTIFICATION OF THE SELECTED STRAIN

Various biochemical tests were performed for the identification and taxonomic characterization of the selected strain. Antibiotic sensitivity tests were also done for the selected strain. The strain was sent to MTCC, IMTECH, Chandigarh for further identification. The isolated strain was also identified based on 16S rDNA sequence. 16S rDNA sequence which was amplified via PCR with two degenerate primers. Forward 5' GTTAGCGGCGGACGGGTGAGTA 3'and Reverse 5' GCGATTCCGGCTTCATGCAGGC 3'. Products after PCR amplification were subjected to sequencing, followed by homology analysis. Nucleotide sequences were determined by the ABI Prism 310 genetic analyzer using the big dye Terminator kit (Applied Biosystems) at BioServe India Ltd., Hyderabad. The identity of the sequence was established by comparing the sequences obtained with the 16S rRNA gene sequences in the database using BLAST software (Altschul *et al*, 1990) and the phylogenetic tree was constructed using the dendrogram implemented in CLUSTAL W (http://align.genome.jp).

3.4.1 Template DNA Prepration (Murray and Thompsom, 1980)

DNA isolation was done according to Murray and Thomson (1980). The procedure is as described below:

1. A single cell colony was inoculated into 10ml Luria Broth (HiMedia) and incubated at 37°C overnight with constant shaking at 180 rpm.
2. 1.5ml of the resulting culture was taken in a sterile eppendorf tube and was centrifuged at 6000 rpm for 5 min.
3. The pellet was resuspended in 576µl of sterile Tris-EDTA buffer(pH 8.0)
4. To the above, 30µl of 10% Sodium Dodecyl Sulphate (SDS) and 3µl (20mg/ml) of proteinase K was added to attain a final concentration of 100µg of proteinase K in 0.5% SDS.
5. The contents of the eppendorf tube were mixed well and incubated at 37°C for 1hour.
6. The incubation was followed by the addition of 10µl of 5M sodium chloride and 80µl of Hexadecyltrimethylammonium bromide (CTAB) (10mg/ml).
7. After thorough mixing the tubes were incubated at 65°C for 10 min. in a waterbath.
8. The tubes were allowed to cool down to room temperature before adding 720µl of chloroform and 30µl of isoamyl alcohol in the ratio of 24:1.
9. The contents were mixed gently and centrifuged at 10,000 rpm for 10 min.
10. The aqueous layer formed on the top was pipetted out using a blunt end sterile tip and transferred into a fresh sterile microfuge tube.

11. As above collected aqueous layer, a mixture of 375µl phenol: 360µl chloroform: 15µl isoamyl alcohol was added to obtain a ratio of 25:24:1.
12. The contents were mixed thoroughly by inversions and centrifuged at 10,000 rpm for 10 min.
13. The resulting aqueous layer was collected in the same way as above and transferred to a fresh tube.
14. To this aqueous layer 0.6 volumes (~600µl) of isopropanol was added and mixed gently, kept at room temperature for 30 min and centrifuged at 12,000 rpm for 15 min at 4°C.
15. The supernatant was removed and the pellet was washed in cold 70% ethanol.
16. The nucleic acid pellet was air dried to remove the last traces of ethanol and resuspended in sterile Tris-EDTA buffer (pH 8.0).
17. The concentration of DNA thus prepared was estimated specrophotometrically using appropriate dilutions (80-100ng was used as template for PCR reactions)

3.4.2 PCR Reaction Mix

10X PCR buffer	2.5µl
2.5 mM each dNTP mix	2.0 µl
Forward primer(10 picomoles)	1 µl
Reverse primer(10 picomoles)	1 µl
Taq DNA polymerase	1U
Template DNA	1 µl
Distilled water	To the final volume of 25µl

3.4.3 PCR Conditions

Annealing Temperature 58°C

Extension Temperature 72°C

3.4.4 Agarose gel electrophoresis (Sambrook *et al*, 2001)

1. Agarose gel with a concentration of 0.8% was prepared for electrophoresis of the PCR products.
2. 5µl of the PCR products was loaded on to the gel and electrophoresed at 80 volts until the migrating dye (Bromophenol blue) had traversed two-third distance of the gel. The 500 bp DNA ladder (Genei, Bangalore) was used as the marker.
3. The gel was stained in freshly prepared 0.5 mg/ml Ethidium bromide solution for 10 min.
4. The gel was viewed on a UV- Transilluminator, and image captured with the help of Digi Doc system (Bio-Rad).

3.5 SUBMERGED FERMENTATION (SmF)

Bioprocess for production of lipase by *Bacillus smithii* BTMS 11 under Submerged Fermentation was optimized using Minimal Salts media as described below.

3.5.1 Minimal salts media

Minimal salts medium mentioned in section 3.2.2 was used unless otherwise specified, to study the lipase production by *Bacillus smithii.* The contents were mixed thoroughly, autoclaved at 121°C for 30 min and cooled to room temperature before inoculation.

3.5.2 Inoculum Preparation:

For the preparation of inoculum, a loopful of cells from the freshly grown culture (ZoBell's Marine Agar slant) of *B.smithii* was transferred to a 5ml ZoBell's marine broth (ZB) and incubated at RT (28 ± 2°C) on a rotary shaker. After 18 hrs of incubation this preculture was again transferred to a 40 ml ZB in a 250 ml conical flask and incubated at room temperature (RT) on a rotary shaker at 180 rpm. Later after 18 hrs of incubation cells were harvested by centrifugation at 6000 rpm for 5 min at 4°C. The sedimented cell pellet was resuspended in 10ml of 0.8% physiological saline (5x 10^8 cfu /ml) and used to inoculate the production media.

3.5.3 Inoculation and Incubation

Minimal salts medium mentioned earlier under section 3.5.1 was inoculated with 2% (v/v) of bacterial inoculum as prepared as mentioned under section 3.5.2, and incubated on a rotary shaker at 28 ± 2°C (RT) at 170 rpm. After the desired period of incubation (48 hrs arbitrarily selected), the enzyme was recovered from the production broth. This was the general procedure followed for submerged fermentation unless mentioned otherwise.

3.5.4 Recovery of enzyme

The fermented media was centrifuged at 10,000 rpm at 4°C for 15 min. The clear supernatant obtained after centrifugation was used as crude enzyme, and assayed for lipase activity and protein content as mentioned under section 3.2.6.2 and 3.2.6.3 respectively.

3.6 PROCESS OPTIMIZATION STUDIES

Various process parameters, which influence lipase production by *B.smithii* were evaluated to effect maximal enzyme production using minimal salts media. Strategy adopted for the optimization was to evaluate the effect of each parameter on lipase production independently and then optimize the significant parameter using statistical methods. The parameters studied included:

3.6.1 Substrate

The substrate optimisation studies were done in two different media namely Minimal Salts media and ZoBell's Marine broth using 12 different lipid sources were added as source of carbon. The various substrates tested includes the following:

1. Olive oil
2. Gingely oil
3. Sunflower oil
4. Coconut oil
5. Castor oil
6. Groundnut oil
7. Tween 20
8. Tween 80
9. Tributyrine
10. Palm oil

11. Soya bean oil

12. Mustard oil

The substrates were added to the medium at of 0.5% (v/v). Media preparation, inoculation, incubation and enzyme recovery was done as mentioned in section 3.6. Culture supernatant obtained was taken as a crude enzyme for assaying lipase activity and protein which was determined as mentioned in sections 3.2.6.2 and 3.2.6.4. The various concentrations (0.1 to 2.5% (v/v)) of the lipid substrate supporting maximum lipase activity were also checked to determine the appropriate concentration of the substrate that can support maximum lipase production. The effect of various combinations of gingelly oil along with coconut oil on lipase production was evaluated at various ratios namely 1:1, 1:2 and 2:1. This approach was aimed to check the effect of one substrate on the other on lipase production in minimal media.

3.6.2 Incubation Temperature.

Optimal incubation temperature for maximal lipase production by *B. smithii* was evaluated by incubating the inoculated minimal media at 28°C$\pm$ 2 (Room Temperature (RT)), 35°C, 40°C, 45°C and 50°C with gingelly oil as a lipid substrate for 48 hrs and determining the enzyme activity. Medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.5. Culture supernatant was taken as a crude enzyme and lipase activity was determined as mentioned in section 3.2.6.2

3.6.3 pH optimization

Optimal medium pH that support maximal enzyme production by *B. smithii* was determined by adjusting the pH of the medium to various levels i.e., 3, 4, 5, 6, 7, 8, 9, 10 and 11 with 1N HCl or 1N NaOH. Medium preparation, inoculation, incubation, and enzyme recovery were performed as detailed under sections 3.5. Culture supernatant was taken as a crude enzyme and lipase activity was determined as mentioned in section 3.2.6.2.

3.6.4. Additional Carbon source:

Requirement for additional carbon source besides lipid substrate for maximal lipase production was evaluated using different sugars and polysaccharides. Ten different carbon sources viz: Glucose, Fructose, Sucrose, Maltose, Lactose, Xylose, Mannose, Glycerol, Mannitol and Starch were tested. The additional carbon sources were sterilized separately and were added to the production media at the rate of 2g/l. Medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.5. Culture supernatant was taken as a crude enzyme and lipase activity was determined as mentioned in section 3.2.6.2.

3.6.5. Additional Nitrogen source.

Impact of additional nitrogen source on lipase production was evaluated using different nitrogen sources as an additional nitrogen source along with the lipid substrate provided in the media. The additional nitrogen sources were added to the production media at the rate of 2g/l.Twelve different nitrogen sources, both organic and inorganic were used for the study.

Organic nitrogen sources included Casein, Soybean meal, Yeast extract, Beef extract, Peptone and Tryptone. Inorganic nitrogen sources used were $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl, $NH_4H_2PO_4$, $(NH_4)_2HPO_4$, and Urea. Medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.5. Culture supernatant was taken as a crude enzyme and lipase activity was determined as mentioned in section 3.2.6.2.

3.6.6. NaCl Concentration.

Impact of NaCl concentrations on the lipase production was checked by adding different concentrations of NaCl namely 0.5%,1%,2%, 3%, 4% ,5%, 6%, 7%, 8%, 9% and 10% (w/v) to the production media with already optimized parameters. Medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.5 respectively. Culture supernatant was taken as a crude enzyme and lipase activity was determined as mentioned in section 3.2.6.2.

3.6.7. Effect of Calcium ions on lipase production.

Impact of calcium ions on maximal lipase production was determined by supplementing production medium with different molar concentration of $CaCl_2.2H_2O$ namely 0.1mM, 0.2 mM 0.3 mM, 0.4 mM, 0.5 mM, 1 mM, and 5 mM. Gingelly oil was used as lipid substrate. Medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.5. Culture supernatant was taken as a crude

enzyme and lipase activity was determined as mentioned in section 3.2.6.2.

3.6.8. Effect of Magnesium ions on lipase production.

Effect of Magnesium ions on lipase production was checked by incorporating magnesium at different molar concentration of $MgSO_4.7H_2O$ (1mM, 2 mM, 3 mM, 4 mM, 5 mM, 10 mM, and 50 mM) in the medium. Medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.5. Culture supernatant was taken as a crude enzyme and lipase activity was determined as mentioned in section 3.2.6.2.

3.6.9. Inoculum concentration

Optimal inoculum concentration that supports maximal enzyme production was evaluated using different concentrations of bacterial inoculum. Inoculum was prepared as mentioned under section 3.5 and was added to the production media at the concentrations ranging from 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10% (v/v) respectively. Medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.5. Culture supernatant was taken as a crude enzyme and lipase activity was determined as mentioned in section 3.2.6.2.

3.6.10. Time Course Experiment.

A time course experiment was conducted after optimization of various process variables with optimized parameters in minimal media viz: Gingelly Oil (1.5% (v/v), pH (8.0), Temperature

(Room Temperature), Glucose additional Carbon Source (0.5% (w/v), Soybean meal additional Nitrogen source (0.2 %(w/v), NaCl (0.5 %(w/v), Ca^{2+} ions in the form of $CaCl_2.2H_2O$ (0.1mM), Mg^{2+} ions in the form of $MgSO_4.7H_2O$ (2mM), Inoculum Concentration of 3% (v/v) and Incubation Time of 72 hrs. Medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.5. Culture supernatant was taken as a crude enzyme and growth, lipase activity, protein and protease activity was determined as mentioned in section 3.2.6.

3.7 OPTIMISATION OF BIOPROCESS VARIABLES FOR LIPASE PRODUCTION BY *B.smithii* - STATISTICAL APPROACH

3.7.1 Plackett – Burman experimental design

Plackett–Burman design, an efficient technique for medium component optimization (Naveena *et al.*, 2005), was used to pick factors that significantly influence lipase production, and to obtain a smaller, more manageable set of factors. It is proved to be a valuable tool for the rapid evaluation of the effects of the various medium components. Eleven variables representing ten nutritional components and initial pH of the medium were used. For each nutrient variable, a high (+) and a low (-) concentration was tested. The main effect of each variable upon lipase activity was estimated as the difference between both averages of measurements made at the high level (+) and at the low level (–). A 12-run Plackett–Burman design (Plackett & Burman, 1946) was used to screen eleven factors, and the experimental

responses were analyzed by the method of least squares to fit the following first-order model:

$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_5X_5+.....\beta_{11}X_{11}$, where Y was the predicted response (Lipase yield), β_0, β_1, β_2 $.....\beta_{11}$ were the regression coefficients, and X_1, X_2..... X_{11} were the coded levels of the independent variables (Table 3.1 & 3.2).

Plackett- Burman design was applied for the statistical optimization of the production of lipase under submerged fermentation by *B.smithii*. Minimal media containing each of the nutrients as per the matrix design at the corresponding pH was formulated and sterilized at 121^0C for 30 min. Prepared media was inoculated and the flasks were incubated at the appropriate temperatures and the enzyme was extracted after 18 and 30 hrs respectively, and the enzyme activity was estimated as mentioned in the section 3.2.6.2. Statistical significance of the model equation was determined by Fisher's test, and the proportion of variance explained by the modelwas presented by the multiple coefficient of determination, R^2 value. Design Expert (Version 7.1.0; STATEASE Inc., USA) was used for the matrix design and interpretations. A validation run was performed to confirm the results after getting the Plackett-Burman results.

Table 3.1 Levels of factors chosen the Plackett and Burman design for lipase production by *B. smithii*

Variable Code	Variable	Levels	
		+1	-1
A	Gingelly Oil (%(v/v))	2	1
B	pH	9	8
C	Temperature (^{o}C)	30	25
D	Glucose (% (w/v))	1	0.2
E	Soybeanmeal (%(w/v))	0.5	0.2
F	$(NH_4)_2SO_4$ (%(w/v))	0.5	0.2
G	NaCl (%(w/v))	1	0.2
H	Inoculum(%(v/v))	4	2
J	$CaCl_2.2H_2O$(mM)	0.1	0.05
K	$MgSO_4.7H_2O$(mM)	3	1
L	Incubation time (hrs)	30	18

Table 3.2 Plackett - Burman design matrix for lipase production

Run	Coded variable level										
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*J*	*K*	*L*
1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1
2	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1
3	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
5	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1
6	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1
7	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1
8	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1
9	-1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1
10	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1
11	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1
12	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1

3.8 ENZYME PURIFICATION

Lipase produced by *B.smithii* under SmF was purified 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.

3.8.1 Ammonium Sulphate Precipitation

Ammonium sulphate (SRL) required to precipitate lipase enzyme was optimized by the addition of various concentrations of ammonium sulfate (20%, 40%, 60%, 80% and 90% saturation) to the crude extract.

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

(b) After complete dissolution of ammonium sulphate the solution was kept at 4°C for over night

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

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

3.8.2. Dialysis

The precipitate obtained after ammonium sulphate precipitation was further dialysed against a buffer in order to remove the ammonium sulphate from the precipitate, as detailed below.

(a) The precipitated protein was resuspended in minimum quantity of 0.1 M Tris HCl buffer (pH 8)

(b) Dialysed in the pretreated dialysis tube (cut of value 12,000kDa) against 0.01 M solution of Tris HCl buffer of pH 8 for 24 hrs, at 4°C with 6 changes of buffer and assayed for lipase activity, protein content and specific activity as mentioned in 3.2.6

3.8.2.1 Pre-treatment of Dialysis Tube.

Dialysis tube is 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 12,000 kDa or greater. The method for the treatment dialysis tube (Sigma –Aldrich USA) is as follows.

(a) Washed the tube in running water for 3-4 hrs

(b) Dipped in 0.3% (w/v) solution of Sodium sulphide at 80°C for 1 minute

(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)

3.8.3 Ion Exchange Chromatography.

Active fraction obtained after ammonium sulphate fractionation followed by dialysis was further purified by ion-exchange chromatography using the anion exchanger DEAE-cellulose (Sisco Research Laboratories Pvt Ltd, India) as the column material.

3.8.3.1 Activation of DEAE- Cellulose

The following method was adopted for the activation of DEAE- Cellulose

(a) 10g of DEAE- Cellulose (SRL) was socked in Phosphate buffer (pH 7, 0.01M) and fine particles were removed by decanting.

(b) It was then suspended in 1M NaCl for over night.

(c) Decanted Sodium Chloride solution and washed several times with distilled water in sintered glass funnel using vacuum filtration, until the pH of washings became neutral.

(d) It was equilibrated in Phosphate buffer of pH 7 (0.01M) by repeated washing with the same.

3.8.3.2 Standardisation of binding pH of lipase to DEAE-Cellulose

The pH at which the enzyme binds at its maximum to the anion exchanger was standardised by eluting enzyme solution after incubating with DEAE- cellulose equilibrated to each pH. DEAE-Cellulose was activated by following the method

described in section 3.8.3.1, suspended in distilled water and equilibrated to each pH using 0.1M buffers of, Citrate–Phosphate buffer (pH 3 to 6), Tris-Hcl buffer (pH 7-8) and Carbonate-bicarbonate buffer (pH 9 & 10). One ml of diluted sample of 60-80% 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. Added 2 ml of 0.4M NaCl and incubated for 2 hrs to elute the bound protein from the DEAE-Cellulose. Supernatant collected was centrifuged at 10000 rpm for 10 min to remove fine particles and assayed for lipase activity and protein content.

3.8.3.3 Purification Using DEAE-Cellulose column

DEAE-Cellulose activated as described in section 3.8.3.1 was carefully packed in XK16/26 column (Amersham Biosciences) without trapping any air bubble. The column (20 x 1.5 cm) was equilibrated with Phosphate buffer of pH 8.0 (0.1M) for overnight.

3 ml of dialysed sample with protein content of 1.5 mg/ml was applied to the pre-equilibrated DEAE-cellulose column. After the complete entry of sample to the column the unbound proteins were washed with Tris HCl buffer of pH 8.0 (0.1M) 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. 5 ml fractions were collected and protein content was estimated by measuring the absorbance at 280nm. Peak fractions from the column were pooled and lipase activity, protein content and specific activity were estimated as mentioned

in the section 3.2.6. Yield and fold of purification was calculated as described in section 3.8.4.1.

3.8.4 Analytical Methods*

Lipase activity, protein content and specific activity were determined as described earlier in sections 3.2.6 and expressed as U/ml, mg/ml and U/mg protein respectively.

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

Yield of protein and enzyme activity of each fraction 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.

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

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

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

3.9 CHARACTERISATION OF PURIFIED ENZYME

Lipase purified by ammonium sulphate fractionation, dialysis and ion exchange chromatography was further characterised for their biophysical and biochemical properties like molecular mass determination, zymogram profile, enzyme kinetics etc. as described in the following sections.

3.9.1 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.

3.9.1.1 Reagents for Polyacrylamide Gel Electrophoresis

1) **Stock acrylamide solution (30:0.8)**

Acrylamide (30%)	60g
Bis - acrylamide (0.8%)	1.6g
Distilled water (DW)	200ml

Stored at 4°C in amber coloured bottle.

2) **Stacking gel buffer stock (0.5 M Tris-HCl pH 6.8)**

Tris buffer 6g 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 36.3g

Titrated to pH 8.8 with 1M HCl 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	3g
Glycine	14.4g

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	-	3g
Glycine	-	14.4g
SDS	-	1g

Dissolved and madeup to 1L with DW

Prepared in 10X concentration and stored at 4°C

6) Sample buffer for Native PAGE

0.0625 M Tris-HCl (pH 6.8)

10% (v/v) Glycerol (optional)

0.01% Bromophenol blue

Prepared in 2X concentration and stored at 4°C

7) Sample buffer for Reductive SDS PAGE

0.0625 M Tris-HCl (pH 6.8)

10% (v/v) Glycerol (optional)

2% SDS

0.1M Dithiothreitol

0.01% Bromophenol blue

Prepared in 2X concentration and stored at 4°C

8) Sample buffer for Non-Reductive SDS PAGE

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

0.01% Bromophenol blue

Prepared in 2X concentration and stored at 4°C

9) SDS (10%)

1g in 10ml DW

10) Sucrose (50%)

5g in 10ml DW

Autocalved at 121°C for 15 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 Native – PAGE

Separate markers from Sigma Aldrich, USA were used.

Bovine Serum Albumin	10 µl	(MW (M_r) 66000)
Chick albumin	10 µl	(MW (M_r) 45000)
Carbonic anhydrase	5 µl	(MW (M_r) 29000)
Lactalbumin	10 µl	(MW (M_r) 14200)

Markers were mixed with 65µl of Native 1X sample buffer, and 30µl of marker mix was loaded on to the gel.

14) Protein Marker for SDS – PAGE

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

Phosphorylase b	MW (M_r) 97000
Bovine Serum Albumin	MW (M_r) 66000
Ovalbumin	MW (M_r) 45000
Carbonic anhydrase	MW (M_r) 30000
Trypsin inhibitor	MW (M_r) 20100
α-Lactalbumin	MW (M_r) 14400

3.9.1.2 Native- Polyacrylamide Gel Electrophoresis (Native-PAGE)

3.9.1.2.1Gel Preparation

1) Resolving gel (10%)

Acrylamide : bis-acrylamide (30: 0.8)	10ml
Resolving gel buffer stock	3.75ml
Ammonium persulphate (APS)	a pinch
Water	16.25ml
TEMED	15µl

2) Stacking gel (2.5%)

Acrylamide : bis- acrylamide (30: 0.8)	2.5ml
Stacking gel buffer stock	5 ml
Ammonium persulphate (APS)	a pinch
Water	12.5 ml
TEMED	15µl

3) Sample buffer (1X)

Native PAGE sample buffer (2X)	1ml
50% Sucrose	0.4 ml
DW	0.6 ml

3.9.1.2.2 Sample preparation

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

3.9.1.2.3 Procedure

1. Cleaned and assembled the gel plates
2. **Resolving gel** - Added all the components except APS in to a beaker, mixed gently (without bubble formation) and

finally added APS. Immediately poured the mixture into the cast and poured a layer of butanol over the gel (to make the surface of the gel even and to avoid contact with air, since oxygen prevents polymerisation) and allowed to solidify at least for 1hr.

3. **Stacking gel-** Added the components of stacking gel and finally added APS, mixed gently and poured the contents into the cast. Immediately inserted the comb between the glass plates. Allowed it to solidify at least for 30 min.
4. Gel was placed in the electrophoresis apparatus, and upper and lower reservoir was filled with reservoir buffer for Native PAGE
5. Pre run the gel at least for 1hr at 80V
6. Loaded the gel with the protein sample
7. Run at volt of 80 till it entered the resolving gel
8. When the dye front enters the resolving gel the current was increased to 100 V.
9. Stopped the current when the dye front reached 1cm above the lower end
10. Removed the gel from the cast and stained for at least 1hr in the staining solution
11. Destained till the bands became clear and observed under a transilluminator

3.9.1.3 Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Purified protein was subjected to reductive or non-reductive SDS-PAGE ie with or without β-mercaptoethanol. Low molecular weight marker of Amersham Pharmacia was used as

molecular mass standard and molecular weight of lipase was determined using Quantity One Software of BioRad.

3.9.1.3.1 Reductive SDS-PAGE

3.9.1.3.1.1 Gel Preparation

1) Resolving gel (10%)

Acrylamide –bis acrylamide(30: 0.8)	10ml
Resolving gel buffer stock	3.75ml
10% SDS	0.3ml
Ammonium persulphate (APS)	a pinch
Water	15.95ml
TEMED	15µl

2) Stacking gel (2.5%)

Acrylamide –bis acrylamide(30: 0.8)	2.5ml
Stacking gel buffer stock	5 ml
10%SDS	0.2ml
Ammonium persulphate (APS)	a pinch
Water	12.3 ml
TEMED	15µl

3) Sample buffer (1X)

SDS-PAGE sample buffer (2X)	1ml
50% Sucrose	0.4 ml
DW	0.6ml

3.9.1.3.1.2 Sample preparation

Added 100µl of 1X sample buffer to lyophilized sample (purified by ion exchange or Preparative gel electrophoresis), mixed well, boiled for 5 min in a water bath, cooled to room temperature, and 30µl sample and 5µl low molecular weight markers were loaded on to the gel

3.9.1.3.1.3 Procedure

Procedure followed for electrophoresis and staining was essentially same as described in section 3.9.1.2.3 with the exception that the reservoir buffer used was that of SDS-PAGE.

3.9.1.4 Non reductive SDS- PAGE

3.9.1.4.1 Gel preparation

Resolving and Stacking gel was prepared as described in section 3.9.1.2. 3

1) Sample buffer (1X)

Sample buffer for Non- Reductive SDS PAGE (2X)	1ml
50% Sucrose	0.4 ml
DW	0.6ml

3.9.1.4.2 Sample preparation

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

3.9.1.4.3 Procedure

Procedure followed for electrophoresis and staining was essentially the same as described in section 3.9.1.2.3 with the exception that the reservoir buffer used was that of SDS-PAGE.

3.9.1.5 Activity Staining for Detection of Lipase Activity

Electrophoresis of the purified lipase was conducted according to Diaz et al (1999) using the fluorogenic substrate 4-Methylumbelliferyl butyrate (MUF-butyrate) (Sigma, St. Louis, USA). For activity staining, after the run, the SDS- PAGE gel was soaked for 30 min in 2.5% TritonX-100® at room temperature, briefly washed in 50mM Tris buffer, pH 8, and covered by a solution of 100μM methylumbelliferyl butyrate (diluted with 50 mM Tris buffer from a stock of 25 mM solution) and incubated for a short period at room temperature. Activity bands were observed under UV illumination. Following zymogram analysis gel was stained with Coomassie Brilliant Blue R®-250 for visualizing protein bands.

3.9.2 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 3.2.6.2. The enzyme solution used was 0.2 ml of diluted sample and the substrate pNPB was prepared in the respective buffer of each pH. The buffer systems used included, Citrate–Phosphate buffer (pH 3 to 6), Tris-HCl buffer (pH 7.0 and 8.0), Carbonate-bicarbonate buffer (pH 9 and 10). Enzyme activity and Relative activity were calculated as described in section 3.2.6.2 and 3.9.12.2.

3.9.3 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 7.0 after incubating the enzyme in different buffer systems of pH 3-10 for varying periods of incubation (1, 6, 12, 24 hrs) and at 4°C.

Purified enzyme as 0.2 ml aliquot was incubated in 1.8 ml of different buffer systems, Citrate–Phosphate buffer (pH 3 to 6), Tris-HCl buffer (pH 7.0 and 8.0), Carbonate-bicarbonate buffer (pH 9.0 and 10.0). After incubation the sample was assayed for lipase activity and enzyme activity and residual lipase activity were calculated as mentioned in the section 3.2.6.2 and 3.9.12.2.

3.9.4 Optimal Temperature for Lipase Activity

Optimum Temperature for enzyme activity was determined by incubating 0.2ml of purified enzyme with the lipase assay reaction mixture at different temperature at the temperature (30°C , 40°C , 50°C , 60°C , 70°C and 80°C) and the enzyme activity and relative activity were calculated as described in section 3.2.6.2 and 3.9.12.2.

3.9.5 Enzyme stability at different temperatures

Temperature stability of purified enzyme was determined by incubating the enzyme sample at various temperatures ranging from 30-80°C and the enzyme assay was conducted at 30 min, 1hr, 2hr, 4hr, 6hr, 8hr, 10hr, 12hr and 24hr of incubation. Enzyme activity of the sample kept at 4°C was taken as control. Enzyme activity and residual lipase activity were calculated as mentioned in the section 3.2.6.2 and 3.9.12.2.

3.9.6 Effect of various chemical agents on Lipase Activity

Effect of various chemical agents on the purified enzyme was determined in order to classify the enzyme depending on the inhibition pattern. Chemical agents like SDS (Sodium Dodecyl Sulphate), Phenylmethylsulphonyl fluoride (PMSF), Ethlene diamine tetra acetic acid (EDTA), Diethyl pyrocarbonate

(DEPC), β-Mercaptoethanol and H_2O_2 were added to the enzyme assay mixture and the residual enzyme activity was measured. Residual enzyme activity and expressed in percentage (section: 3.9.12.1).

3.9.7 Substrate specificity

Ability to hydrolyse various synthetic substrates by the purified enzyme was evaluated by conducting enzyme assay with various p-Nitrophenyl derivatives namely p-Nitrophenyl Acetate, p-Nitrophenyl Butyrate, p-Nitrophenyl Caprylate, p-Nitrophenyl Laurate and p-Nitrophenyl Palmitate. with 10 mM solution of respective substrates prepared in acetoniterile. Enzyme activity was expressed in U/ml.

3.9.8 Positional specificity

Positional specificity of the lipase was determined by use of a method modified from that described by Lesuisse *et al.* (1993). 40 mg of pure triolein was sonicated in 2 ml of 0.1 M Tris-HCl (pH 8.0) containing 1 mM calcium chloride for 3 min. The enzyme sample (200µg) was then added and the reaction mixture was incubated at 65°C for 2 hrs. After incubation, the reaction products were extracted by the addition of 1.5 ml diethyl ether. The extract was concentrated by evaporation and applied to a silica gel plate (Merck Co.). Plates were developed with a 96:4:1 mixture (by volume) of chloroform/acetone/ acetic acid. The spots of glycerides and fatty acids were visualized by exposure to iodine vapor.

3.9.9 Kinetic studies

Purified enzyme was subjected to kinetic studies towards determining the K_m and V_{max}. K_m (substrate concentration at which the reaction velocity is half maximum) and V_{max} (velocity maximum) of the enzyme reaction was determined by incubating 0.2ml of the purified enzyme in different concentrations (10-200μM) of p-nitrophenyl Butyrate. Enzyme assay was essentially the same as described in section 3.2.6.2. The initial velocity data was plotted as the function of the concentration of substrate by the linear transformation of the Michaelis-Menten equation (Lineweaver- Burk Plot) and usual non-linear curve fitting of the Michaelis-Menten equation for the calculation of K_m and V_{max} of the reaction.

3.9.10 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 3.9.12.1). The metals studied included 1, 5, 10 mM final concentrations of Sodium chloride, Calcium chloride, Magnesium sulphate, Zinc sulphate, Potassium chloride, Cupric sulphate, Ferric chloride, Manganese chloride, nickel chloride, and Cobalt chloride respectively.

3.9.11 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 30 min and assaying the residual activity (Section 3.9.12.1).

Organic solvents studied included, Methnol, Isopropanol, Ethanol and Hexane (1, 2, 5, 10 %) respectively.

3.9.12 Analytical Methods*

Lipase activity, protein content and specific activity were determined as described earlier in section 3.2.6.

3.9.12.1 Residual Activity

Residual activity is the percent enzyme activity of the sample with respect to the enzyme activity of the control sample.

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

3.9.12.2 Relative Activity

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

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

* All the experimental data were statistically analysed using Microsoft Excel.

3.10 Application Studies

Detergent industry is the prime field which consumes the largest share of the microbial lipase produced. The addition of lipases to detergent formulations has been investigated in the context of removal of fat stains. Removal of oil or fatty deposits by lipase is attractive owing to its suitability under washing conditions. To be a suitable additive in detergents, lipases should be both thermophilic and alkalophilic and capable of functioning in the presence of the various components of washing powder formulations. Hence, an

attempt was done to check the compatibility of *B.smithii* lipase as an additive to modern detergents and its effect on wash performance.

Apart from detergent industry, lipases are today the chief consumables in the esterification and transesterification reactions because of their regiospecificity and steriospecificity. *B. smithii* lipase was also checked for its ability to carry out estrification reactions.

3.10.1 Evaluation of enzyme for use in detergent formulations.

In order to determine the suitability of enzyme for use in detergents, its compatibility was studied in various detergent ingredients including surfactants and commercial detergents, by directly incorporating these into the assay mixture.

3.10.1.1 Effect of Surfactants on lipase activity.

Effects of various surfactants on enzyme activity were evaluated by incubating the enzyme with each surfactant for 30 minutes and assaying the residual activity. Various surfactants checked included Tween 80, Triton- X -100, Gum Arabic and PEG 6000 (0.1%, 0.5%and 1%).

3.10.1.2 Commercial detergent compatibility of enzyme

The stability of the enzyme in the presence of commercial detergent was determined using various detergents which included Ujala washing powder, Surf excel, Ariel compact, Henko stain champion, Tide, Sunlight extra bright with colour lock, at concentration 7 mg/ml (w/v). Enzymes already present in the detergent and soap solutions were first heat inactivated by boiling for 10 min and to the 50ml, solution of detergent, 2ml of purified enzyme sample was added and incubated for 3 hrs at RT. Samples were taken out after 3hr and the residual activity was determined as described in section 3.9.13.1.

3.10.1.3 Wash performance studies

Wash performance analysis of purified lipase (60-80% ammonium sulphate precipitate with enzyme activity of 482 U/ml) was studied on white cotton cloth piece (5 cm x 5 cm) stained with oily stain. The stained cloth pieces were taken in separate flasks and subjected to the following wash treatment studies.

1. 100ml heat inactivated detergent (7 mg/ml) + Stained cloth piece
2. 100ml heat inactivated detergent (7 mg/ml)+1ml enzyme solution+ Stained cloth piece
3. 100ml distilled water + 1ml enzyme solution + Stained cloth piece
4. 100ml distilled water + Stained cloth piece

After 30 min of incubation at RT in a water bath shaker, the cloth pieces were taken out rinsed with tap water, dried and visual examination was done to check the effectiveness of stain removal (Sreeja, 2005).

3.10.2 Esterification ability of *B.smithii* lipase

B.smithii lipase was investigated for its ability to carry out a variety of esterification reactions between fatty acids (C4: 0 to C18: 1) and methanol in n-hexane (Gulati *et al*, 2001). For a typical reaction, 100 mM of the fatty acid was mixed with 50 mM of the alcoholic donor methanol in 4 ml n-hexane in 15-ml screw capped vials The reaction mixture was incubated at 50°C for 24 hrs at 150 rpm. Ester synthesis was quantified by titrating the remaining fatty acids in the reaction mixture with 0.1N NaOH. The ester content was quantified by calculating the residual fattyacid amount in the reaction mixture and expressed in terms of per cent conversion of fatty acid to ester.

Chapter 4

RESULTS

4.1 Primary Screening

Results obtained for the primary screening of lipase producing bacteria is presented in Table: 4.1. Out of the 280 isolates screened with two different substrates, namely Tween 80 and Tributyrine the positive isolates were short listed on the basis of the clearing zones (Fig:4.1) (Tributyrine agar plates) or precipitated zones (Tween 80 agar plates) (Fig:4.2) noticed in the plates. 58 isolates were recognized as positive for lipase production.

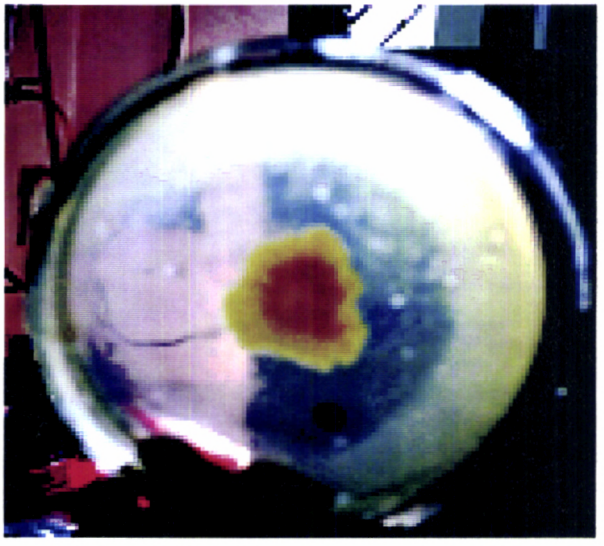


Figure 4.1 Lipase activity in Tributyrine agar plate

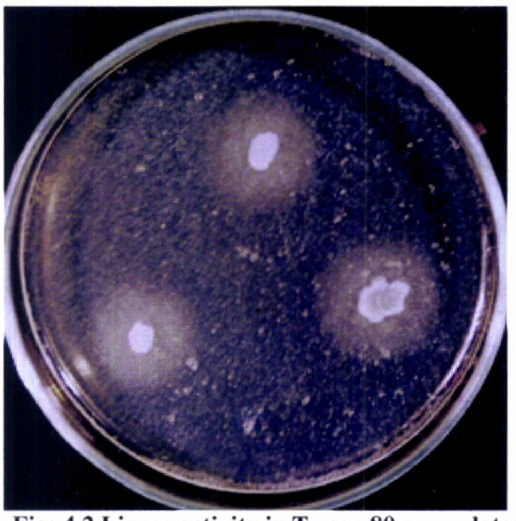


Fig: 4.2 Lipase activity in Tween 80 agar plates

Table: 4.1 58 isolates selected after the primary screening in Tween-80 and Tributyrine plates

SL.NO	ISOLATE NO	TRIBUTYRIN	TWEEN-80
1	BTMW18	+	+
2	BTMW60	+	+
3	BTMW63	+	+
4	BTMW84	+	+
5	BTMW273	+	+

6	**BTMW275**	+	+
7	**BTMW276**	+	+
8	**BTMW326**	+	+
9	**BTMW332**	+	+
10	**BTMW343**	+	+
11	**BTMS3**	+	+
12	**BTMS22**	+	+
13	**BTMS42**	+	+
14	**BTMS44**	+	+
15	**BTMS76**	+	+
16	**BTMS82**	+	+
17	**BTMS118**	+	++
18	**BTMS142**	+	+
19	**BTMS149**	+	+
20	**BTMS162**	+	+
21	**BTMS197**	+	+
22	**BTMB14**	+	++
23	**BTMB15**	+	+
24	**BTMB16**	++	+
25	**BTMB17**	+	+
26	**BTMB18**	+	+
27	**BTMB19**	+	++
28	**BTMB63**	+	+
29	**BTMB66**	+	+
30	**BTMB67**	+	+
31	**BTMB 77**	+	++
32	**BTMB 78**	+	++
33	**BTMB 80**	++	+
34	**BTMB 83**	+	++
35	**BTMB 84**	+	++
36	**BTMB 85**	+	+
37	**BTMB 86**	+	+

Table 4.1 Continued

38	BTMB 87	+	+
39	BTMB 88	+	++
40	BTMB 90	+	+
41	BTMB 91	+	+
42	BTMB 94	+	++
43	BTMB 95	+	+
44	BTMB 99	+	++
45	BTMB 101	+	+
46	BTMS4	++	++
47	BTMS5	++	+
48	BTMS6	++	+
49	BTMS7	++	+
50	BTMS11	++	+
51	BTMS12	++	+
52	BTMS13	++	+
53	BTMS14	+	+
54	BTMS16	++	+
55	BTMS20	++	+
56	BTMW2	+++	+
57	BTMW7	+++	+
58	BTMW11	+	+

+ 2 - 8mm clearing zone/halo

++ 1 - 1.5 cm clearing zone/ halo

+++ 1.5 - 2 cm clearing zone /halo

4.2 Secondary Screening:

Secondary screening was done with the 58 isolates selected from the primary screening to discriminate specific lipase producing strains from that of the esterase producers. Out of the 58 isolates, 10 cultures which showed fluorescent halos around the wells (Fig: 4.3) in the Rhodamine plates under UV irradiation were selected (Table: 4.2) for final screening.

Table: 4.2 Isolates positive in Rhodamine-B -Olive Oil Plates

ISOLATES			
MB 16	MB 17	MB 19	MB 77
MB 85	MB 86	MB 94	BTMS11
S 82	S 162		

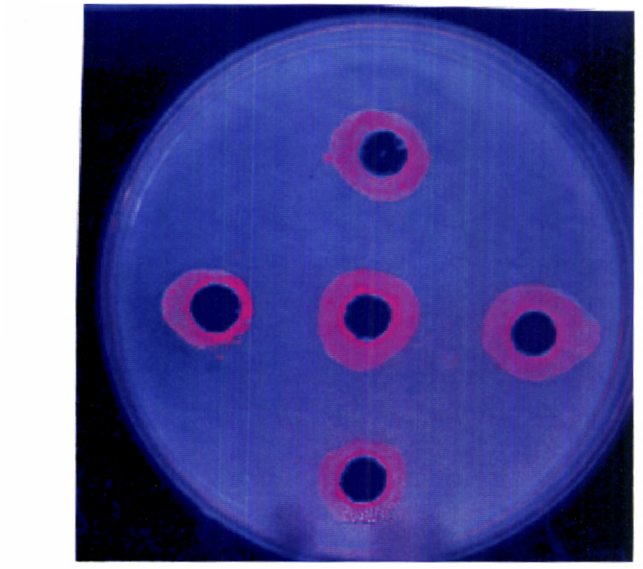
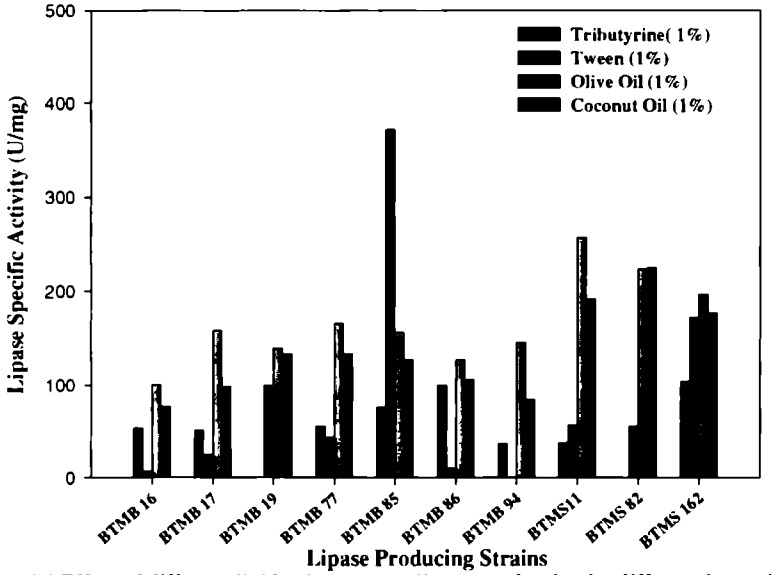


Fig: 4.3 Lipase activity in Rhodamine-Olive oil agar plates

4.3 Final screening

Among the selected 10 cultures after secondary screening were checked for their ability to grow and produce lipase in four different substrates and in two different media, namely Minimal medium and ZoBell's marine broth. All the cultures showed good growth and lipase production with all the four lipid substrates tested (Fig 4.4 and 4.5). BTMS-11 was selected for the further studies on the basis of the higher specific activity and the ability to grow in the Minimal media and to use the lipid substrate as the sole source of carbon for the growth and enzyme production. BTMS-11 was found to have promising growth both minimal media and ZoBell's Marine broth with Olive oil, Olive oil being a complex lipid substrate. The organism also showed good growth and enzyme production with coconut oil, Tween 80 and Tributyrine as a source of carbon.



4.4 Effect of different lipid substrates on lipase production by different bacterial cultures in minimal media. study conducted by incubating different bacterial isolates in Minimal media with different lipid substrates at 1%(v/v) level. Lipase activity estimated after 48 hours of incubation

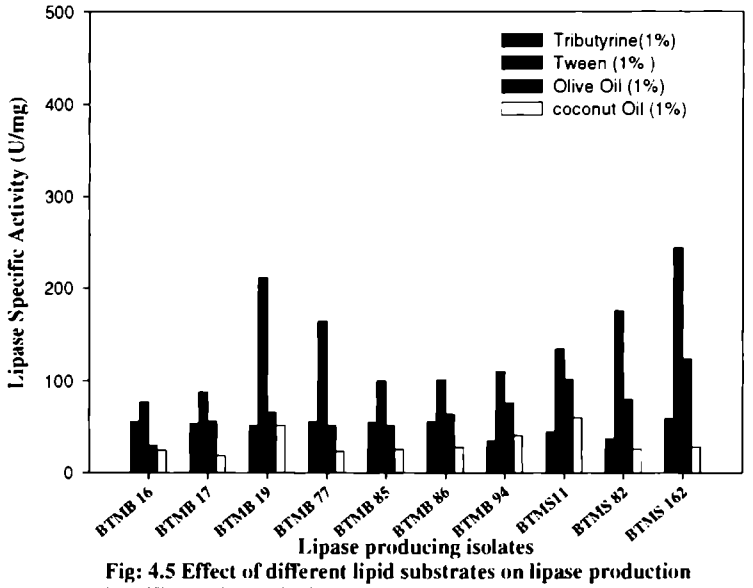


Fig: 4.5 Effect of different lipid substrates on lipase production by different bacterial isolates in Zobells media. Study conducted in Zobells Marine Broth with different lipid substrates at 1% level. Lipase activity estimated after 48 hours of incubation at RT.

4.4 Identification of the selected strain:

The organism was identified as *Bacillus smithii* by MTCC, IMTECH, Chandigarh (Table:4.3). The molecular identification of the bacterial strain was done by ribotyping using partial 16S rRNA gene. A portion of the 16S rRNA gene (~1200 bp) (Fig: 4. 6) was amplified from the genomic DNA and the amplicon was subjected to sequencing, followed by homology search and analysis.

4.4.1 Phylogenetic tree construction

The identity of the sequence was established using BLAST software (Altschul *et al.*, 1980). The obtained sequence showed 90% identity(Accession No:EF581007) with already available sequences of *Bacillus smithii* in the GenBank. Phylogenetic tree was constructed using the dendrogram method implemented in CLUSTAL W (Fig.4.7, 4.8). Partial nucleotide sequence obtained after sequencing was 830 bp.

Table:4.3 Morphological, Cultural and biochemical characteristics of Bacillus smithii BTMS 11

Test	Results
Colony morphology	
Configuration	Circular
Margin	Entire
Elevation	Raised
Surface	Smooth
Pigment	Light orange
Opacity	Opaque
Gram's Reaction	+ve
Cell Shape	Rod
Size(um)	3 0-5.0
Arrangement	-
Spore(s)	+ve
Endospore	+
Position	Sub-Terminal
Shape	Ellipsoidal
Sporangia bulging	Non Swollen
Growth at Temperature	
4°C	-
10°C	-
15°C	+
25°C	+
30°C	+

7°C	+
42°C	+
45°C	+(w)
55°C	-
65°C	-
Growth at pH	
pH 4.0	-
pH 5.0	-
pH 6.8	+
pH 8.0	+
pH 9.0	+
pH 11.00	+
pH 12.00	+
Growth on NaCl (%)	
2.0	+
4.0	+
5.0	+
7.0	+
10.0	+
Growth under anaerobic condition	+ve
	-
Growth on MacConkey agar	
Biochemical Tests	
Indole test	-
Methyl red test	-
Voges Proskauer test	-
Citrate Utilization	+
Gas production from glucose	-
Casein hydrolysis	+(w)
Esculin hydrolysis	+(w)
Gelatin hydrolysis	-
Strach hydrolysis	+
Urea hydrolysis	-
Nitrate reduction	-
H_2S production(TSI agar)	-
Catalase test	+

Oxidase test	+
Lysine decarboxylase	+
Ornithine decarboxylase	+
Dnase activity	-
Tween 80 hydrolysis	+
Tween 60 hysdrolysis	-
Adonitol	-
Arabinose	-
Dulictiol	-
Dextrose	+(w)
Lactose	-
Maltose	+
D-maninitol	+
Surose	+(w)
Xylose	-

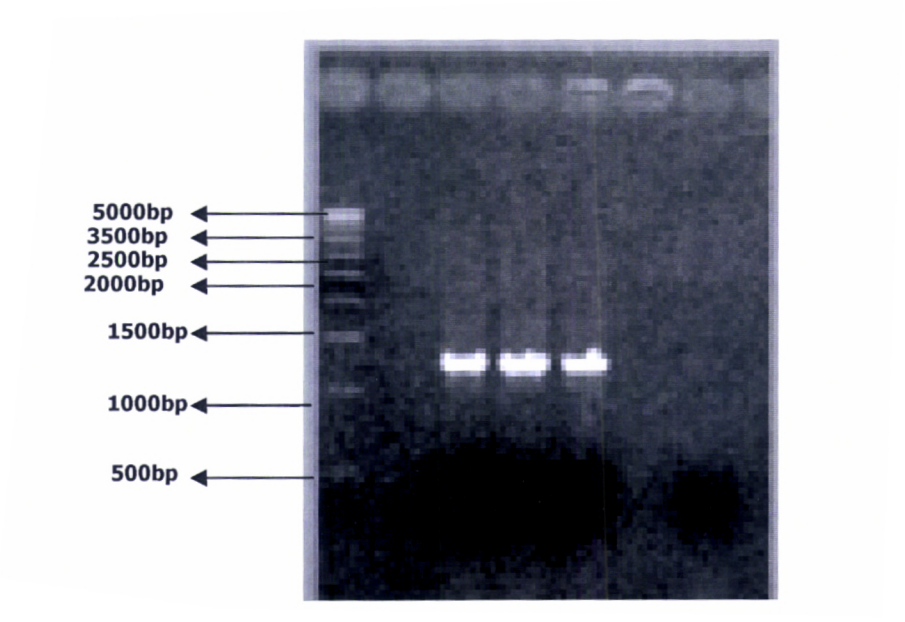


Fig. 4.6 PCR amplicon of 16s rDNA segment amplified with specific primers from the genomic DNA of *B.smithii* BTMS 11

AACTGAGATGCTCCGGTAACCGGAGCTAATACCGGATAACATTTTCTCTTGCATGAAGAGAAC
ATTGAAAGATGGTTTCGGCTATCACTTACAGATGGGCCCGCGGTGCATTAGCTAGTTGGTGA
GGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAA
AGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTA
GGGAAGAACAAGTACAAGAGTAACTGCTTGTACCTTGACGGTACCTAACCAGAAAGCCACGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGG
CGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAG
GGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGAAAGCGGATCCCGTGTAGCGTGAATG
CGTAAGATGTGGAGACCCAGTGCAGGCGCTTTGGTTTACGACCGAGCCAGCTGGACACAGAT
AGATCCTGTATCCCGAACATATCAGGTAAGTTCGCTTATCGCGTACCTAGCTGCGGATCGTCA
ATACTAGATACGGCGCACGGACTGTATCACACAACTCGTGCTCTCCTATACTCCGCATAGTCG
TTTCGTGATGTTCACCATTCTTCTGTGACAGATTTCTGCCTGTGAGCTCTTTATTGTAAAAATT
TNNNCGATGGTCCCCTAGA

Fig: 4.7 *Bacillus smithii* partial 16S rDNA gene Sequence

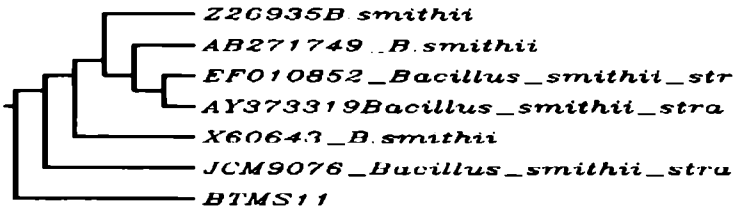


Fig. 4.8 Dendrogram representing the relationship of *Bacillus smithii* BTMS 11 with other reported *B.smithii*

4.5 Submerged Fermentation (SmF): Production of Lipase by *B.smithii* BTMS 11

Lipase production by *B.smithii* BTMS 11 under submerged fermentation was studied using minimal medium and ZoBell's marine broth. Various parameters that influence lipase production were optimized towards maximal lipase production.

4.5.1 Substrates

Twelve different oils were used as a sole lipid substrate in order to select the best substrate that supports maximal lipase production. The results are presented in Fig: 4.9, 4.10 and 4.11, 4.12. It was observed that the bacterium utilised all the twelve different oil substrates provided as a sole source of carbon for its growth and lipase production in both the media tested (4.9 and 4.10). Among all the substrate tested, maximum lipase production was recorded with Gingelly oil (20.23U/ml) followed by Coconut oil (19.00 U/ml), Sunflower oil (17.80U/ml), Olive oil (16.89 U/ml), Castor oil (16.20U/ml), Groundnut oil (15.08U/ml), Mustard oil (14.49U/ml) and Tween 80 (14.37U/ml).Tween 20 (4.25U/ml) showed very less lipase activity compared to other lipid sources in Minimal media (Fig:4.9). In ZoBell's Marine broth, the organism showed very high lipase production with all the lipid substrates checked (Fig: 4.10).Maximum lipase production was observed with Sunflower oil (180 U/ml) followed by Olive oil(156 U/ml), Castor Oil(150 U/ml), groundnut oil(150 U/ml), Tween 20 (149 U/ml) and Gingelly oil(131 U/ml). But taking the production economics into

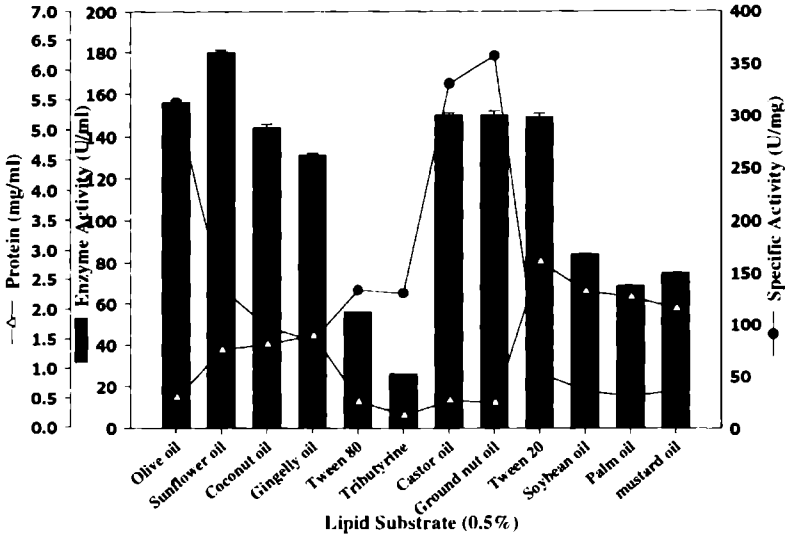


Fig:4.10 Effect of different lipid Substrates on lipase production by *B.smithii* in ZoBell's Marine Broth. Optimization studies conducted in Zobells marine broth with different lipid substrates added to the media at the concentration of 0.5% level and incubated at Room Temperature (28 ± 2) at 170 rpm.

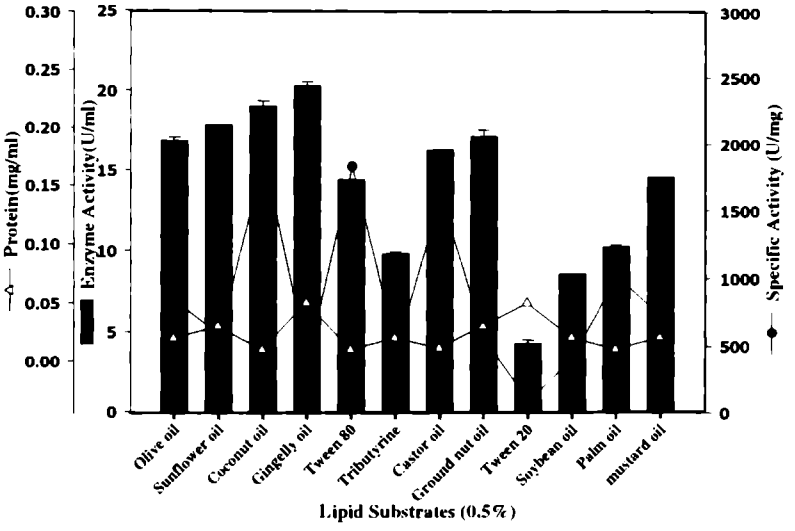
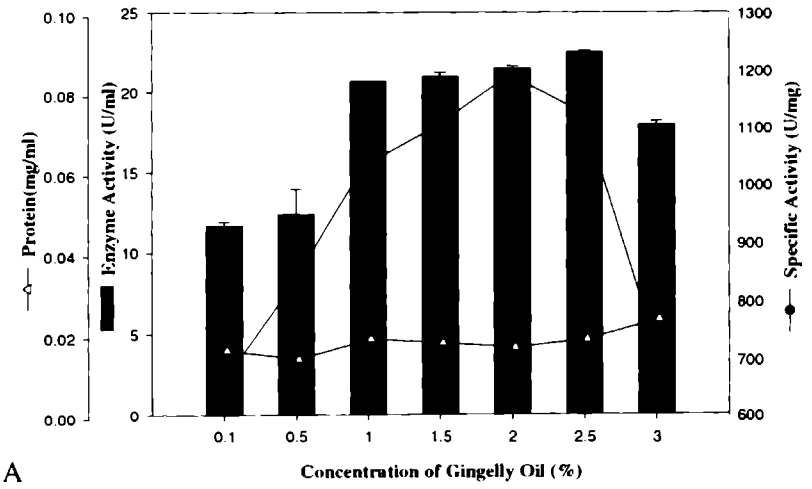


Fig: 4.9 Effect of different lipid substrates on lipase production by *B.smithii* in Minimal media.Optimization studies conducted in Minimal media with different lipid substrates added to the media at 0.5% level and incubated at room temperature at 170 rpm.

Consideration. Minimal media was selected as a basal media for optimization studies. Hence, gingelly oil was selected as the substrate for lipase production by *B. smithii*.

Varying concentrations of gingelly oil (0.5-3%) influenced lipase production by *B. smithii* (Fig:4.11) and thus lipase production increased along with increase in oil concentration to a certain level and later there was a steep decrease in the lipase secretion. Although maximal lipase production was observed with 2.5% of Gingelly oil (25.54 U/ml), almost equal levels of lipase activity could be recorded at 1.5% (24.48U/ml) and 2% (24.11U/ml) concentrations also. Therefore taking into the economy of the production, 1.5% of Gingelly oil was taken as optimum for maximal lipase production.



A

Fig:4.11 Effect of different concentrations of gingelly oil on lipase production by *B.smithii* in minimal media. B.smithii incubated at RT(28±2)^{0}C in minimal media with different concentrations of gingelly oil.

A combination of gingelly oil and Coconut oil, as lipid substrates, in various ratios of concentration (1:1, 1:2, 2:1) was tested for its effect on lipase production (Fig: 4.12). The two substrates were added together in the media such that both the substrates could be used sequentially or simultaneously. But this mixed lipid substrate did not improve enzyme yield compared to that obtained with individual substrates as sole carbon sources. The lipase activity observed for the various ratios tried included 8.56 U/ml, 9.58 U/ml, and 9.95 U/ml respectively for the ratios 1:1, 2:1 and1:2 of Gingelly oil and Coconut oil.

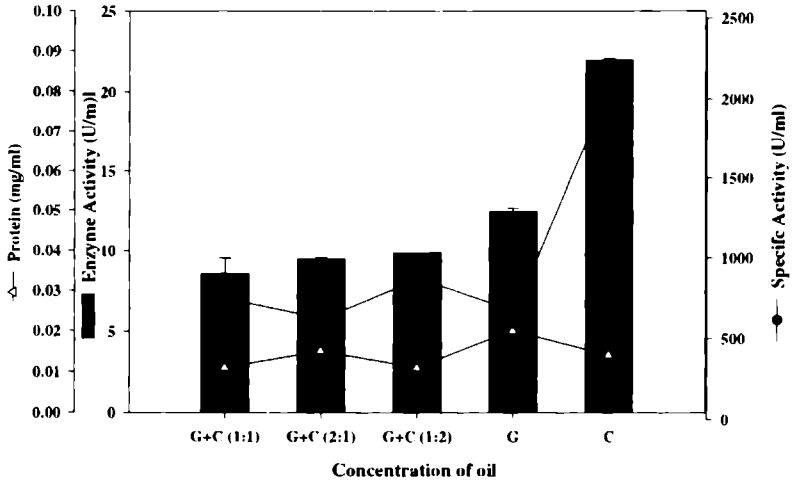


Fig:4.12 Effect of different combination ratios of Sesame and Coconut oil on lipase production by *B.smithii* in minimal media. Study conducted at RT(28±2), 180 rpm. with different combination ratios of (G)Gingelly and (C)Coconut oil. Lipase activity estimated after 48 hours.

4.5.2 Temperature.

From the results presented in Fig: 4.13 it is evident that the bacterium could grow well and produce lipase in minimal salts media supplemented with Gingelly oil and pH 8.0 at all the temperatures studied. However, maximum lipase production was observed at room temperature (28±2) (24.39 U/ml)

Followed by 35°C (23.56 U/ml) and 40°C (17.87 U/ml). Lipase production was recorded even at 45°C (9.07 U/ml) and 50°C (8.66 U/ml) although it was comparatively very low. The ability of the organism to grow and produce maximal enzyme at room temperature is a desirable advantage when industrial production is favoured. Lipase activity showed gradual decrease along with increasing temperature.

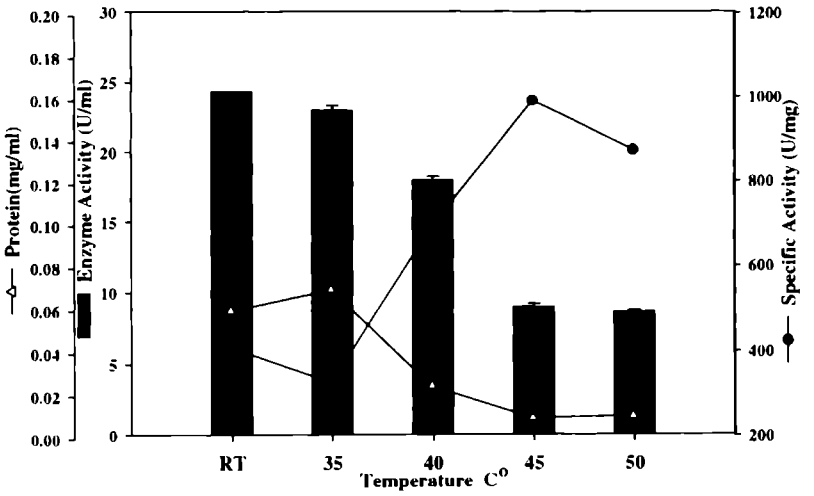


Fig:4.13 Effect of incubation temperature on lipase production by B.smithii in minimal media containing gingelly oil as a lipid substrate. Temperature optimization study conducted by incubating *B.smithii* at different temperatures in minimal media with Gingelly oil (1.5%) and lipase activity estimated after 48 hours.

4.5.3 pH

B. smithii was able to grow at all the pH tested, from pH 2.0 to pH 11.0, in Minimal media containing Gingelly oil (1.5%) (Fig: 4.14). There was no lipase production in spite of considerable growth in the acidic pH up to pH 5. Whereas, lipase activity increased exponentially from pH 6.0 (8.06 U/ml) to pH 8.0 at which a peak value (41.11 U/ml) was recorded. However, the lipase activity declined gradually on further increase in pH 9.0 (34.12 U/ml) to pH 10.0 (28.1 U/ml). The organism showed good pH tolerance and lipase production over a range of pH 6.0 to 11.0. Maximum lipase activity was observed at pH 8.0 and therefore pH 8.0 was taken as optimum for further studies.

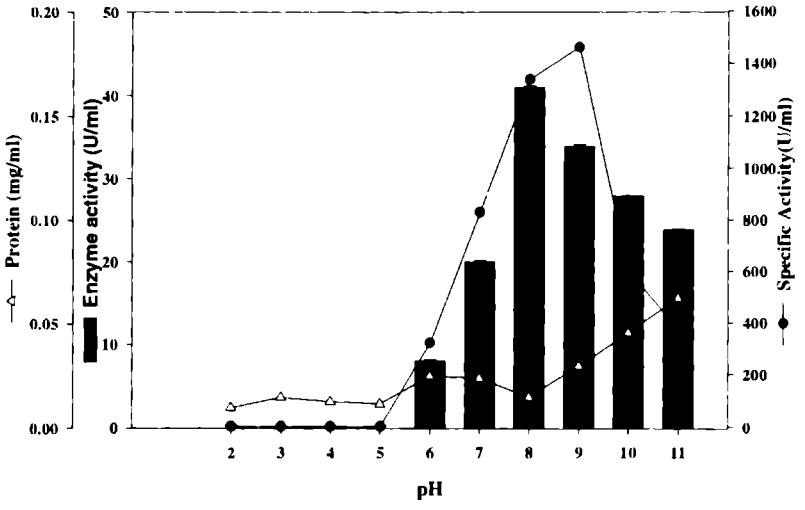


Fig:4.14 Effect of media pH on lipase production by *B.smithii* in minimal media containing Gingelly oil as lipid substrate. pH optimization study conducted in minimal media with different pH, Gingelly oil (1.5%), RT(28±2) at 180 rpm. Lipase activity checked after 48 hours.

4.5.4 Additional Carbon Source.

Among the different sugars and polysaccharides tested as an additional carbon source along with the lipid substrates, except glucose (42.59 U/ml), all other substrates did not favour lipase production in spite of supporting growth compared to the control (32.65 U/ml). No lipase production was observed with maltose as a additional carbon source (Fig: 4.15).Since glucose supported lipase production as additional carbon source, further studies was conducted to determine the concentration of glucose required. Data presented in Fig: 4.16 indicate that lipase production increased along with increase in glucose concentration up to 0.5% (53.93 U/ml) and later steeply decreased at higher concentrations.

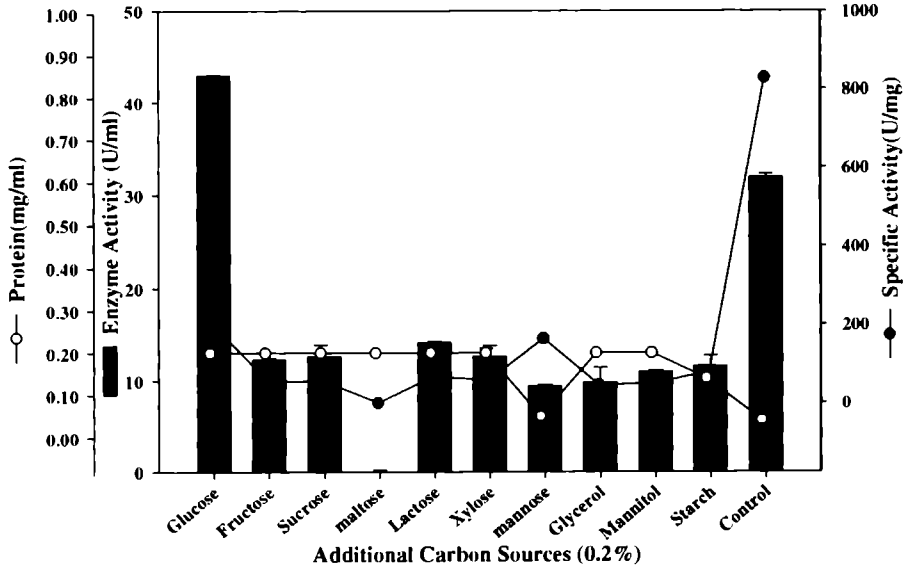


Fig: 4.15 Effect of additional carbon sources on lipase production by *B.smithii* in minimal media containing gingelly oil as lipid substrate. Optimization study conduction in minimal media with 1.5% gingelly oil, RT(28±2), pH 8.0, different sugars and polysaccharides as additional carbon sources at 0.2% level. Lipase activity estimated after 48 hours.

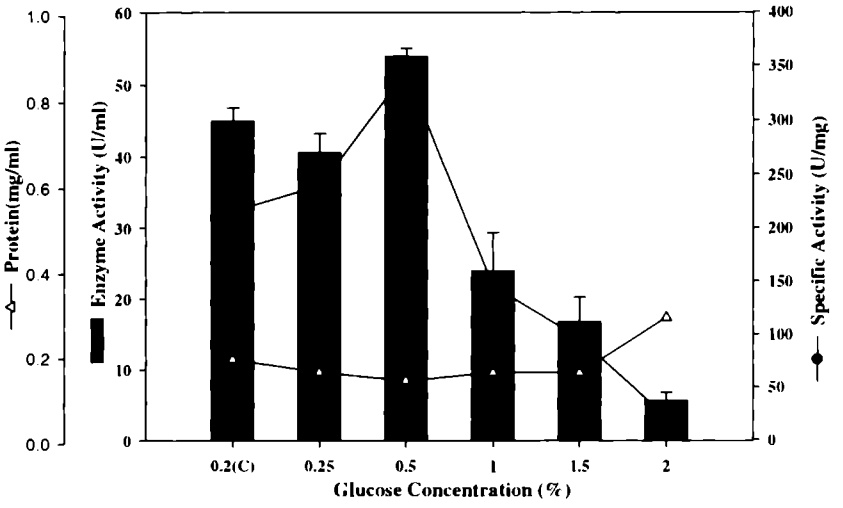


Fig: 4.16 Effect of glucose on lipase production by B.smithii in minimal media containing gingelly oil as a lipid substrate. Optimization study conducted in minimal media, 1.5% gingelly oil, RT(28±2)^{0}C , pH 8.0, 170rpm and different concentrations of glucose. Lipase activity estimated after 48 hours of incubation.

4.5.5 Additional Nitrogen Source.

Addition of various nitrogen substrates as additional nitrogen sources showed rapid increase in growth and lipase production with all the nitrogen sources tested (Fig: 4.17). Among the organic nitrogen sources, 0.2% soybean meal (194.90 U/ml) recorded maximum lipase activity followed by peptone (143.75 U/ml). Similarly Ammonium sulphate (142.13U/ml) and ammonium hydrogen phosphate (111.11 U/ml) also depicted remarkable lipase production. Since soybean meal supported maximal lipase production, optimal concentration of soybean meal was determined by adding different higher concentrations of soybean meal (Fig: 4.18) added along with Gingelly oil were also evaluated for its effect on the lipase production. It was observed that increasing concentration of soybean meal has a repressive effect on lipase production. No significant lipase activity was recorded at higher soybean meal concentrations. So 0.2% of soybean meal was taken as optimum for further studies.

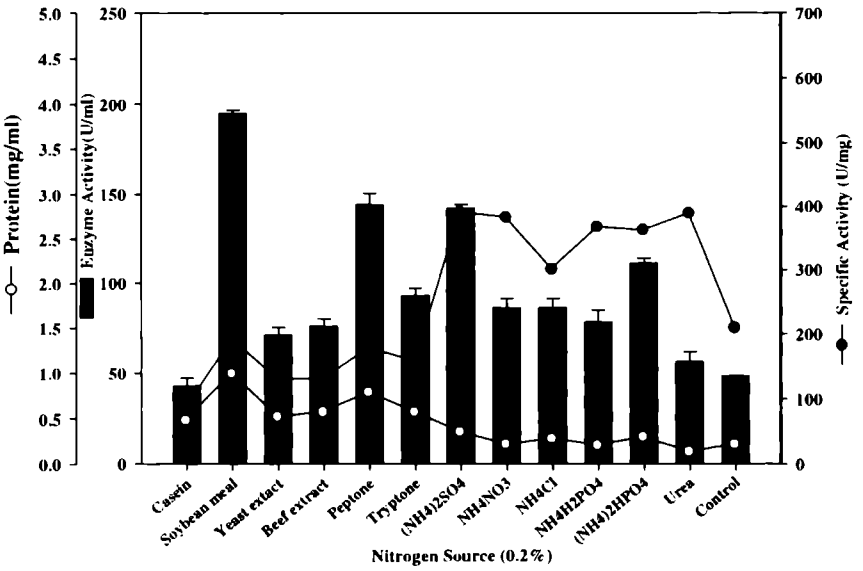


Fig: 4.17 Effect of different nitrogen sources on lipase production by *B.smithii* in minimal media containing gingelly oil as lipid source. Optimization study conducted in minimal media with 1.5% gingelly oil, RT(28±2)^{0}C, pH 8.0, glucose 0.5%, 170 rpm and with 0.2%of different organic and inorganic nitrogen sources. Lipase activity estimated after 48 hours.

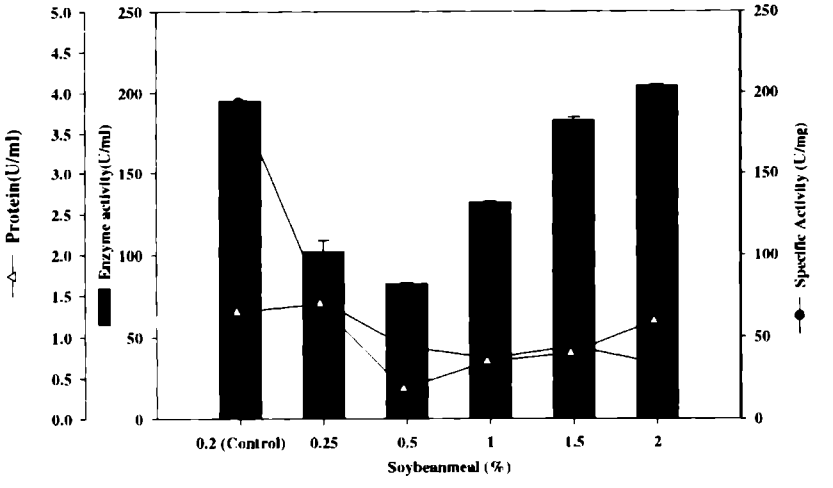


Fig: 4.18 Effect of soybean meal on lipase production by *B.smithii* in minimal salts media containing gingelly oil as a lipid substrate. Optimization studies conducted in minimal media, 1.5% gingelly oil, RT(28±2)^{0}C, pH 8.0 0.5% glucose, 180 rpm and different concentrations of soybeanmeal. Lipase activity estimated after 48 hours of incubation.

4.5.6 Calcium Ion Concentration

Calcium ions are reported to have profound effect on the lipase production. In this respect, different molar concentration of $CaCl_2$ $.2H_2O$ in the media (0.1mM-5.0 mM) supplemented with Gingelly oil as lipid substrate was tested for lipase activity. Results presented in Fig: 4.19 evidence that maximum lipase production was attained with 0.1 mM (Control) concentration of Ca^{2+} (190.51 U/ml) compared to enhanced concentrations of Ca^{2+}in the medium. Lipase activity declined at 0.2mM concentration of Ca^{2+} (68.05 U/ml). Further, increase in molar concentrations of calcium ions showed an inverse relationship with both the lipase production and the growth profile of the organism. The present study indicated that 0.1mM of Ca^{2+} is optimum for the lipase production by *B. smithii* with Gingelly oil as substrate.

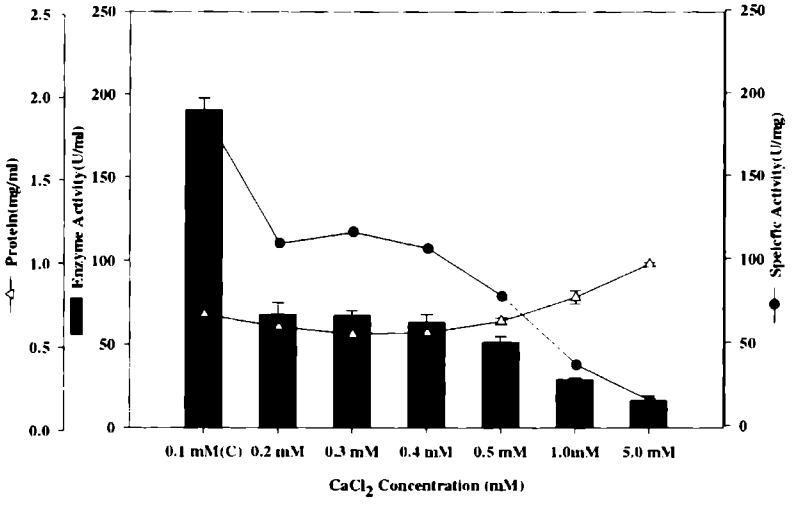


Fig:4.19Effect of Ca2+ ions on the lipase production by B.smithii in minimal media containing gingelly oil as a lipid substrate. Optimization study done in minimal media with 1.5%Gingelly oil, RT(28±2)^{0}C, pH 8.0, 0.5% Glucose, 0.2% soybean meal and different molar concentrations of $CaCl_2$. Lipase activity estimated after 48 hours of incubation.

4.5.7 Magnesium Ion Concentration

Results presented in Fig: 4.20 suggests that maximum lipase activity was recorded at 2mM (Control) of Mg^{2+} ion concentration (194.21 U/ml) followed by 1mM (114.12 U/ml). Lipase activity showed an exponential decrease with increasing concentration of Mg^{2+} ions in the media (94.44 U/ml and 92.59 U/ml respectively for 3mM 4mM). Hence, 2mM of $MgSO_4.7H_2O$ was considered as optimum for the lipase production with Gingelly oil as substrate.

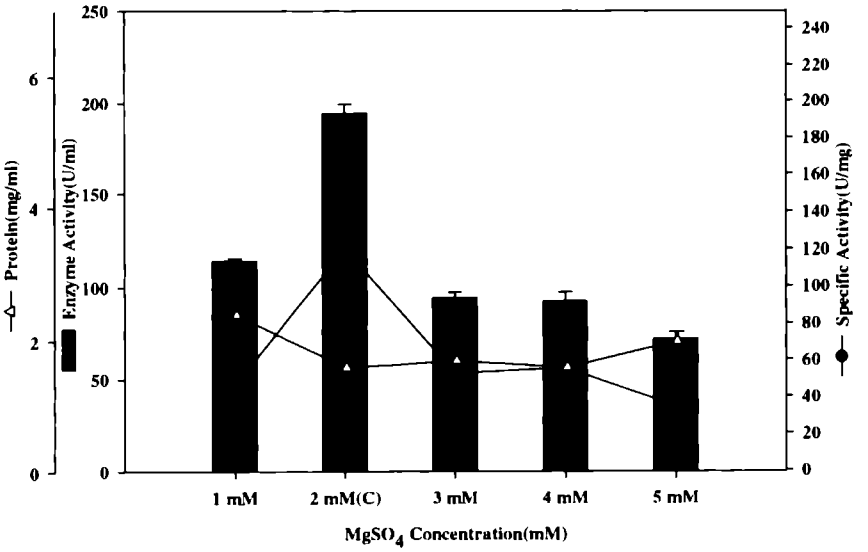


Fig:4.20 Effect of Mg_{2+} ions on lipase production by B.smithii in minimal media containing gingelly oil as a lipid substrate. Optimization studies done in minimal media with 1.5% gingelly oil, RT(28±2)^{0}C, pH 8.0, 0.55 glucose, 0.2% soybean meal, 0.1mM $CaCl_2$, and different millimolar concentrations of MgSO4. Lipase activity estimated after 48 hours of incubation.

4.5.8 Additional NaCl Concentration.

Results obtained in the present study (Fig: 4.21) suggest that *B. smithii* is halotolerant since there was good growth with all the concentrations of the NaCl tested when added as additional source. However, lipase production was maximum at 0.5% of NaCl (223.84 U/ml) followed by 0.2% (187.73 U/ml). A steady decrease in lipase activity was observed along with the increase in the concentrations of NaCl. Nevertheless, lipase production was recorded at considerable level at all the concentrations of NaCl tested

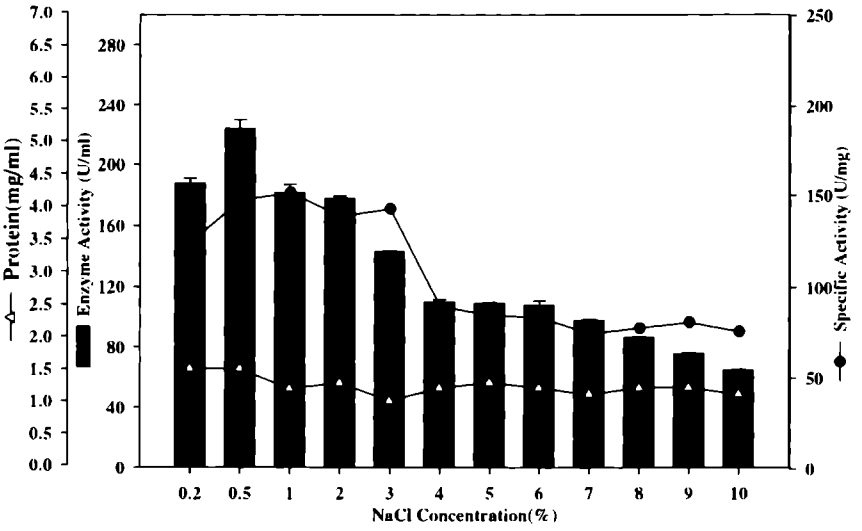


Fig:4.21 Effect of NaCl on lipase production by *B.smithii* in minimal media containing gingelly oil as a lipid substrate.Optimization study conducted in minimal media with 1.5% gingelly oil,RT(28±2)^{0}C, pH 8.0, 0.5% glucose, 0.2% soybean meal, 0.1mM $CaCl_2$, 2mM $MgSO_4$, 170 rpm and different concentrations of NaCl. Lipase activity estimated after 48 hours of incubation.

4.5.9 Inoculum Concentration.

Lipase production showed steady increase along with increase in inoculum concentration up to 3% inoculum (230.09 U/ml) when maximum was recorded (Fig:4.22). Nevertheless lipase production at 4% (220.37 U/ml) and 5% (223.84 U/ml) of inoculum concentration was at considerable level (Fig:4.21). The decline in enzyme activity with increasing inoculum concentration was recorded from 6% (90.04 U/ml) inoculum concentration onwards.

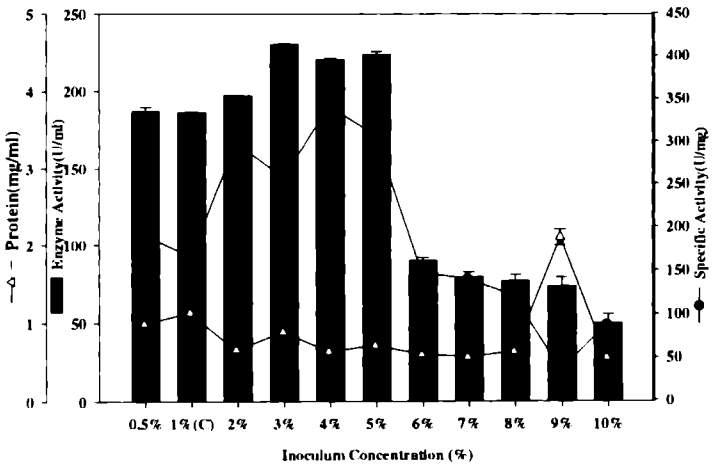


Fig:4.22Effect of inoculum concentration on lipase production by *B.smithii* in minimal media containing gingelly oil as lipid substrate. Optimization studyconducted in minimal media with 1.5% gingelly oil, RT(28±2)^{O}C, pH 8.0, glucose 0.5%, soybean meal 0.2%, $CaCl_2$ 0.1mM, $MgSO_4$ 2mM, NaCl 0.5%, 170 rpm and different inoculum concentrations. Lipase activity estimatedafter 48 hours of incubation.

4.5.10 Time Course study

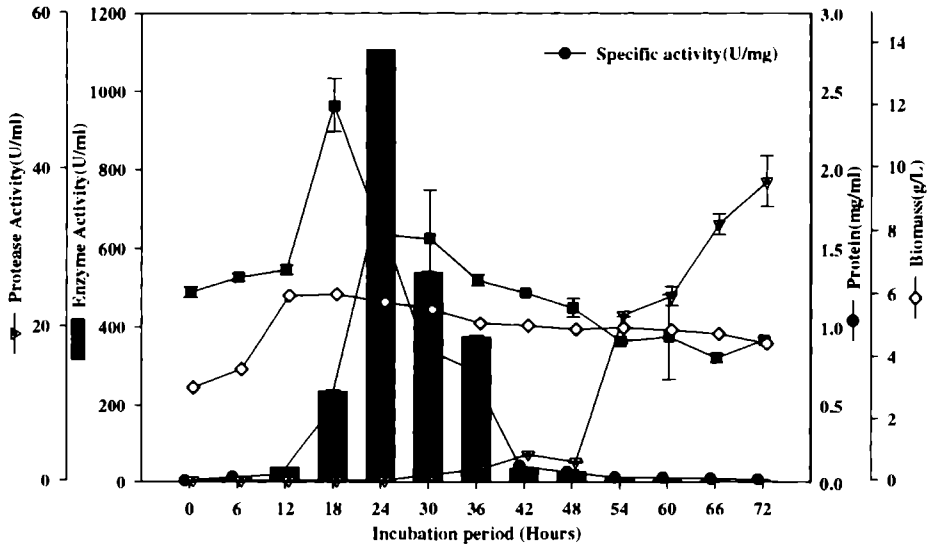


Fig: 4.23 Time course Study for Lipase Production by *B.smithii* using gingelly oil as a lipid source. Time course study done in minimal media under optimized parameters viz: Gingelly oil 1.5%, RT(28±2)^{0}C, pH 8.0, Glucose 0.5%, Soybeanmeal 0.2%, $CaCl_2$ 0.1mM, $MgSO_4$ 2mM, NaCl 0.5%, Inoculum 3% for 72 hours.Lipase activity, specific activity, Protease activity, Cell biomass estimated every 6 hours of incubation

The results presented in Fig: 4.23 for the Time course experiment conducted for 72 hours under optimized condition indicate that the lipase production commenced after12 hours of incubation and reached a peak (Lipase activity of 1108.33U/ml, with a Specific activity of 694.37 mg/ml) at 24 hours. Later the enzyme activity declined rapidly at 30 hours and 36 hours of production. Maximal specific activity and biomass was however recorded at 18 hours which declined gradually. Hence, it is evident that the lipase production is growth associated since the enzyme was secreted in to the medium during the period from the early exponential phase to the early stationary phase.In case of *B.smithii*, lipase started

to appear in the production medium from the 6th hour of growth (12.50 U/ml; 9.09 mg/ml) and increased steadily up to 12th hour (37.50 U/ml; 15.51 mg/ ml) and 18th hour (236.11 U/ml; 177.93) with the simultaneous increase of biomass. Maximum lipase production was achieved at 24th hour (1108.33 U/ml; 694.37 mg/ml). Though there was fairly good production of lipase even at 30th (539.35 U/ml; 343.42 mg/ml) and 36th (375.93 U/ml; 287.47mg/ml) hours, the production appeared to decrease at 42nd (34.72 U/ml; 37.87 mg/ ml) and 48th hours (27.31 U/ml; 22.25 mg/ml) of production. After that only traces of protease production was recorded up to 72nd hour. So the protease production was observed towards the stationary phase.

4.6 Optimisation of process variables for lipase production by *B.smithii* under SmF - Statistical approach

4.6.1 Plackett – Burman Experimental Design

According to the Plackett-Burman experimental design twelve different conditions were run for evaluating the effects of independent variables on the production of lipase under submerged fermentation by the marine *B.smithii*. Each row represents an experiment and each column represents an independent variable. The signs (+) and (–) represent the higher and lower levels of the independent variable under investigation. The results of experimental responses are presented in the Table 4.4.

The estimated effects of individual parameters are presented in Table 4.5. From this, it was inferred that the Gingelly Oil, pH, $CaCl_2$ and Incubation time has a positive influence on lipase production, whereas the temperature, soybeanmeal, Ammonium sulphate, concentrations of NaCl, $MgSO_4$ and inoculum concentration shows a negative effect on lipase production by *B.smithii* under submerged fermentation.

Table 4.4 The result of f plackett – Burman experimental design for optimisation of process variables for lipase production by *Bacills smithii* under SmF

Ex Run	Gingelly Oil(%)	pH	T°C	Glucose(%)	Soybean meal (%)	Am Sulphate (%)	NaCl (%)	Inoculam (%)	Cacl- (mM)	$MgSO_4$ (mM)	Incubation (Hrs)	Enzyme activity(u/m)
1	2	9	25	0.2	0.1	0.5	0.2	4	0.2	1	30	436
2	2	8	30	1	0.5	0.1	0.2	2	0.2	1	30	289
3	2	8	25	0.2	0.5	0.1	1	4	0.05	3	30	250
4	1	8	25	0.2	0.1	0.1	0.2	2	0.05	1	18	80
5	2	9	25	1	0.5	0.5	0.2	2	0.05	3	18	204
6	1	8	25	1	0.1	0.5	1	2	0.2	3	30	128
7	1	8	30	0.2	0.5	0.5	0.2	4	0.2	1	18	33
8	1	9	30	1	0.1	0.1	0.2	4	0.05	3	30	148
9	1	9	30	0.2	0.1	0.1	1	2	0.2	3	18	300
10	2	8	30	1	0.1	0.5	1	4	0.05	1	18	144
11	1	9	25	1	0.5	0.1	1	4	0.2	1	18	156
12	1	9	30	0.2	0.5	0.5	1	2	0.05	1	30	150

Table 4.5 Estimated effects of individual parameters

Code	Parameter	Estimated effects
A	Gingelly oil	157.5
B	pH	75.5
C	Temperature	-28.83
D	Glucose	-32.83
E	Soybean meal	-28.5
F	Ammonium sulphate	-18.5
G	NaCl	-13.17
H	Inoculum	-0.17
I	$CaCl_2$	58.17
J	$MgSO_4$	-29.17
K	Incubation	83.5

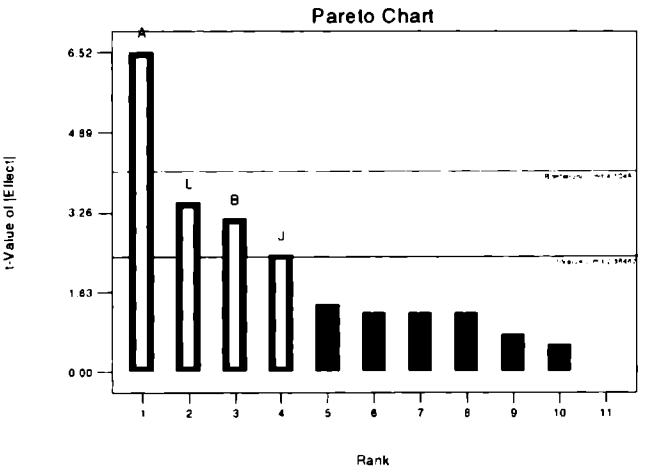


Fig.4.24 Pareto Chart

A graphical representation of the positive (orange bars) and negative (blue bars) effects of the variables tested is presented as a Pareto Chart in Fig.4.24. The significant ones were labeled by respective codes and ranked based on their estimated effects.

On the basis of the experimental values, statistical testing was carried out using Fisher's test for analysis of variance (ANOVA) (Table 4.6). The results were used to fit a first order polynomial equation and the model equation for lipase yield could be written as:

$Y = 191.75 + 78.75\ A + 37.75\ B + 29.08\ I + 41.75\ K$

where Y is yield of lipase, A is Gingelly Oil (%), B is pH of the media, I is the $CaCl_2$ concentration in mM and K is the Incubation period in hrs.

The Model F-value of 17.49, and values of prob > F (<0.05) indicated that the model terms are significant. The test model was statistically significant with a confidence levels above 95%. The quality of fit of the polynomial model equation was expressed by the coefficient of determination (R^2), which equaled 0.9091, indicating that 90.92 % of the variability in the response could be explained by the model.

The adequacy of the model was further examined at additional independent conditions that were not employed to generate the model. It was observed that the experimental and predicted values of lipase production showed good correlation (Table 4.7).

Table: 4.6 Fisher's test for analysis of variance (ANOVA)

Source	Sum of Squares	DF[a]	Mean Square	F Value	p-alue Prob > F
Model	1.226E+005	4	30646.58	17 49	0.0009
A-Gingelly oil	74418.75	1	74418 75	42 48	0 0003
B-pH	17100 75	1	17100.75	9.76	0.0167
J-CaCl	10150.08	1	10150 08	5 79	0 0470
L-Incubation	20916 75	1	20916.75	11 94	0.0106
Residual	12263.92	7	1751 99		
Total Coral	1 349E+005	11			

[a]Degree of freedom — $R^2 = 0.9091$
[b]Significant at "Prob > F" less than 0 05 — Adjusted $R^2 = 0\ 8571$
[c]Difference between experimental and predicted points — Predicted $R^2 = 0.7327$
[d]Total of all information corrected for the mean — Adequate Precision = 13 867

The optimum conditions predicted for the production of 379.08 U/ml lipase were as follows: Gingelly oil (X_1) – (%), pH (X_2) – 9.0, $CaCl_2$(X_3) – 0.2 (mM) and Incubation (X_4) - 30(hrs). The actual experimental value obtained at these predicted conditions was 423.56 U/ml, which was in good agreement with the predicted value.

Table 4.7 Validation of the model

X_1	X_2	X_3	X_4	Y_{exp}	Y_{pred}
2	9.0	0 2	30	423 56	379.08
2	8 0	0 2	29	385 04	311.88
2	9.0	0 17	18	356.0	283 33

4.7 Enzyme purification and characterization

4.7.1 Enzyme purification

The lipase enzyme isolated from *B.smithii* BTMS 11 was purified by ammonium sulphate fractionation followed by anion exchange chromatography on DEAE Cellulose. Lipase could be precipitated with 80% ammonium sulphate saturation.

Table 4.8 Purification of lipase isolated from *B.smithii* BTMS 11

Purification step	Volume (ml)	Total protein (mg)	Total activity (U/ml)	Specific activity (U/mg)	Protein Yeild %	Activity Yeild%	Fold of Purification
Crude extract	760	1900	158330	83	100	100	1
$(NH_4)_2SO_4$	18	25.56	3420	134	1.34	2.16	1.6
DEAE Sepharose	6	2	720	360	0.1	0.45	4.33

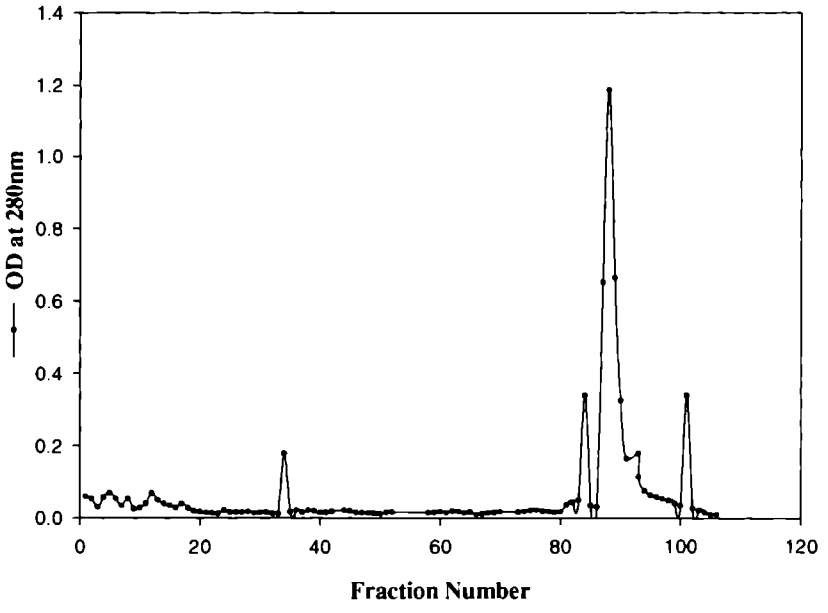


Fig:4.25 Elution Profile of B.smithii Lipase from DEAE Cellulose Coloumn

Table 4.8 summarises the data of the purification steps of the extracellular lipase A purification fold of 4.33 was obtained with ion exchange chromatography. During ion exchange chromatography on DEAE cellulose, the *B smithii* lipase was eluted as a single active peak (Fig.4 25). The elution was carried out with 0.1 M -0.5M NaCl in Tris HCl buffer of pH 8.0 at a flow rate of 5 ml/15 min The active fractions were pooled and dialyzed overnight against Tris HCl buffer (0 01M; pH 8 0).

4.7.2 Enzyme characterization

This purified lipase preparation was used for subsequent studies Various physico-chemical properties such as the molecular size of the protein, temperature and pH optima, substrate specificity, positional specificity, enzyme kinetics, effect of metal ions and effect of organic solvents etc on lipase enzyme were evaluated and are presented in detail below

4.7.2.1 Molecular weight determination

The purity and apparent molecular mass of the lipase preparation was determined by SDS–PAGE (Non-Reductive and Reductive) electrophoresis (Fig 4 26). The purified enzyme showed a single protein band on SDS having a molecular mass of ~ 45 kDa. The zymogram analysis of the gel confirmed the single polypeptide nature of the corresponding band by staining with 4-Methyl Umbelliferyl Butyrate. The results of reductive SDS-PAGE (Fig. 4.27) also shows a single band revealing the single polypeptide nature of the enzyme

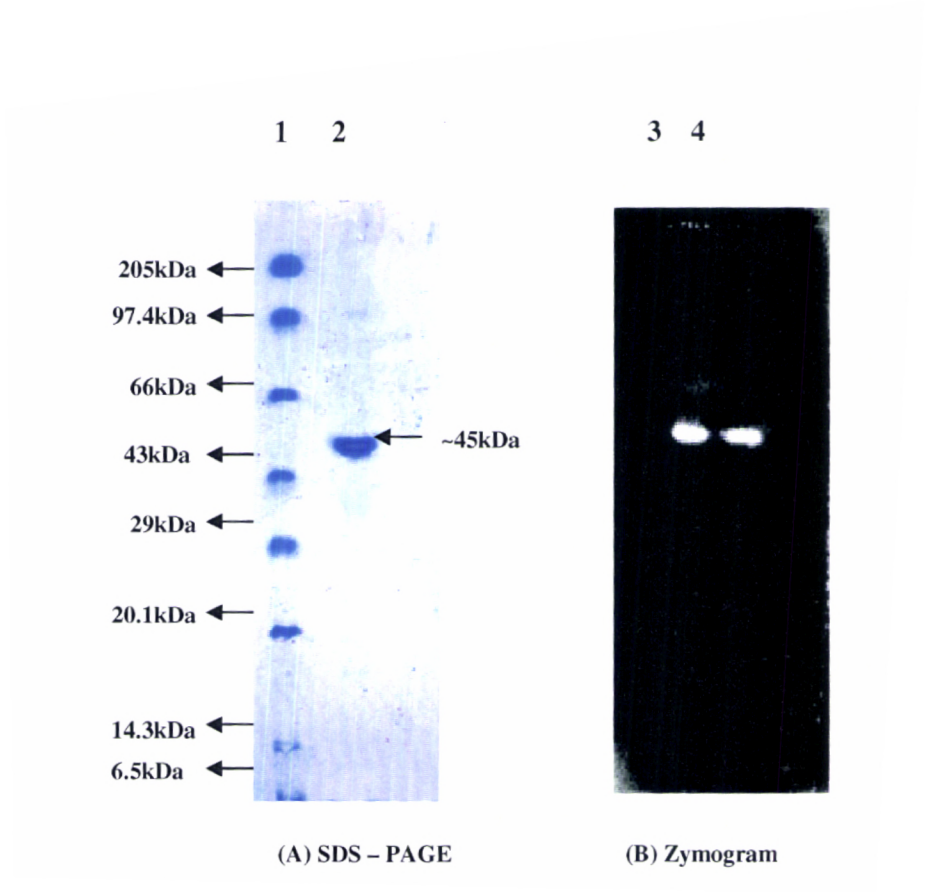


Fig. 4.26 SDS-PAGE (Non-Reductive) analysis of *B.smithii* BTMS 11 Lipase showing (A) Coomassie blue staining and (B) Zymogram analysis

Lane 1 – Protein Marker (kDa)

Lane 2– Ion Exchanged fraction

Lane3– Ammonium Sulphate fraction

(Zymogram with MUF Butyrate)

Lane 4- Ion Exchanged Fraction

(Zymogram with MUF Butyrate)

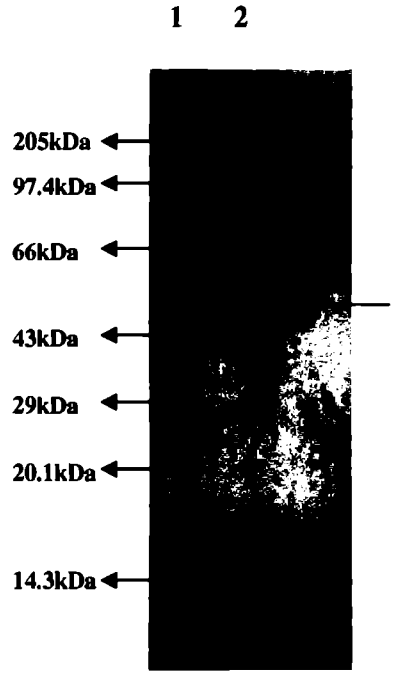


Fig. 4.27 SDS-PAGE analysis of B.smithii BTMS11 Lipase showing Coomassie blue staining

Lane 1 – Protein Marker (kDa)

Lane 2– Ion Exchanged fraction

4.7.2.2 Effect of Temperature on Lipase activity.

Results presented in the Fig.4.28 indicate that enzyme was active over a range of temperature (30^0C to 80^0C) with maximal activity at 50°C. Temperature above 60°C led to a slow decline in enzyme activity. In fact, the lipase activity showed a linear increase along with increase in temperature and particularly, the increase was rapid during 40°C – 50°C. Data depicted in Table: 4.9 on relative activity of lipase at different temperatures indicated clearly the preference for a higher temperature of 50°C for maximal activity.

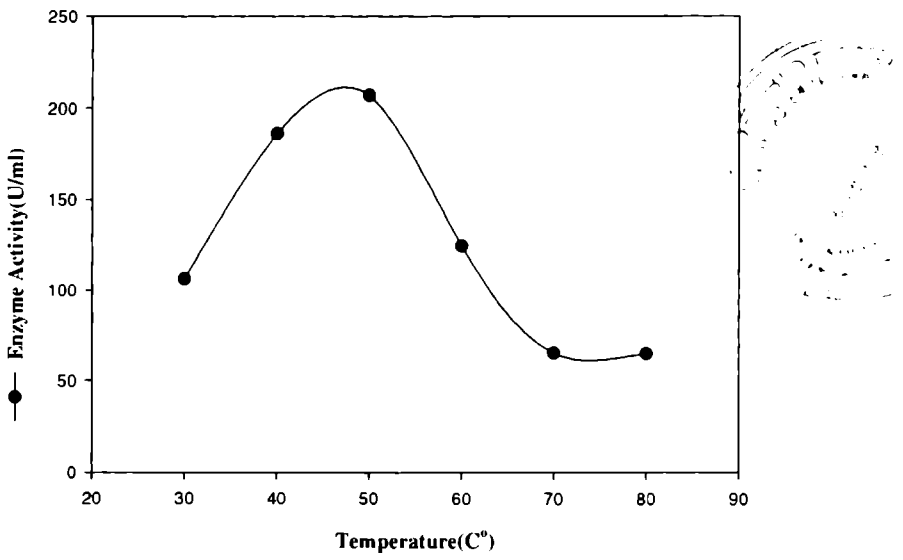


Fig: 4.28 Temperature Optima profile for *B.smithii* Lipase

Table: 4.10 Relative Activity of B.smithii Lipase at different Temperature

Temperature (^{0}C)	Relative Activity (%)
30	51
40	90
50	100
60	61
70	32
80	31

4.7.2.3 Thermostability of lipase.

From the results presented in Fig. 4.29 for the temperature stability studies conducted using purified lipase, it is concluded that the enzyme could retain 50% of activity even after 12 hrs at 30°C to 80°C. enzyme showed a decline in activity after 12hrs.

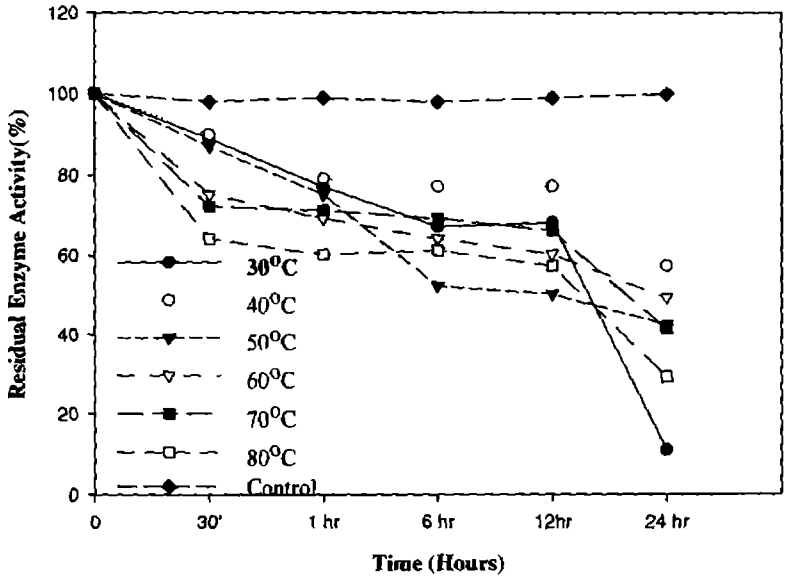


Fig: 4.29Thermostability of *B.smithii* Lipase at different temperature.

4.7.2.4 Effects of pH on Activity and Stability

Results depicted in Fig.4.30 indicate that lipase of *B.smithii* has an optimum pH of 8.0 for maximal activity. In general the lipase was active over a pH range of 7-10 and increase in pH from 7 to 10 recorded proportionate increase in activity from pH 7.0 which reached its peak at pH 8 0 and then showed a steady and gradual decrease with increasing pH. The results suggest that this is an alkaline lipase Where as, enzyme

is totally inactive in the pH below 7. Data presented in Table 4.11 evidence the impact of different pH on relative enzyme activity of lipase. Considering pH 8.0 as optimal for maximal activity, it was observed that the activity declined along with decrease in pH significantly. From the data obtained for the pH stability studies of the lipase (Fig. 4.31), it is inferred that the enzyme is stable over range of pH from 7.0-9.0. However, maximal residual enzyme activity was recorded in the sample incubated in the buffer having pH 8.0.

Table:4.11 Relative Activity profile of *B.smithii* Lipase

pH	Relative Activity (%)
3	0
4	0
5	0
6	0
7	74
8	100
9	78
10	26

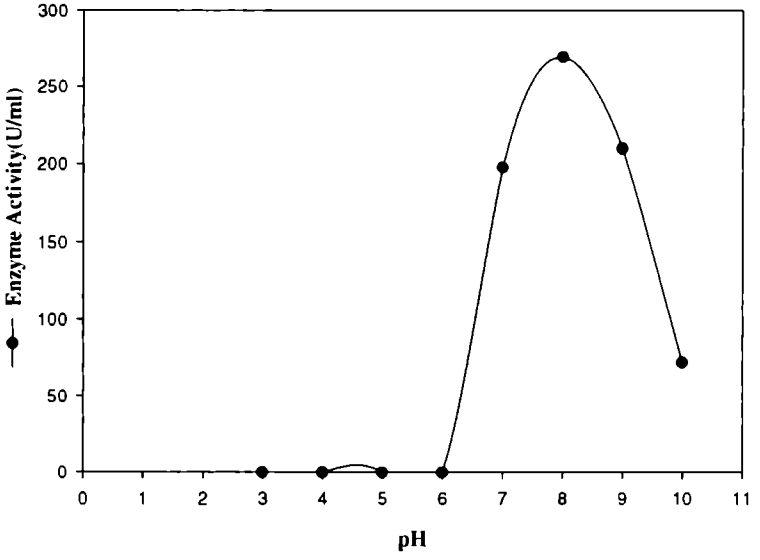


Fig: 4.30 Activity profile of B.smithii Lipase at different pH.

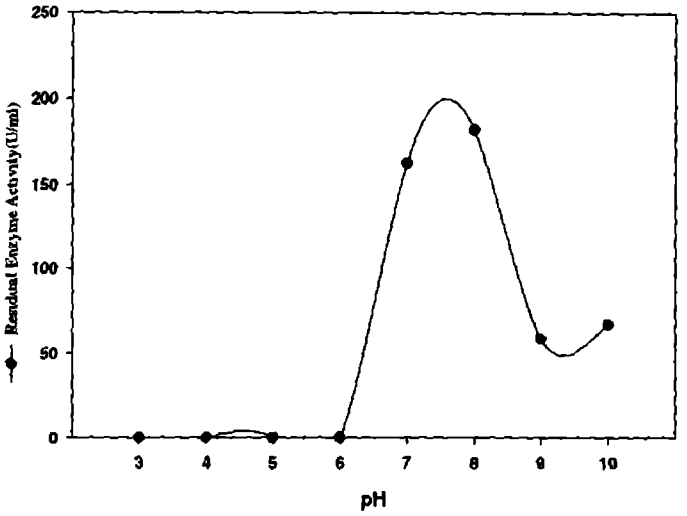


Fig:4.31pH Stability profile of B.smithii lipase

4.7.2.5 Enzyme Kinetics

The reaction kinetics of the purified enzyme was determined from Line weaver-Burk plots (Fig.4.36) with *p*NPB under standard assay conditions (30 min, pH 7.0, 37°C). The enzyme had an apparent K_m value of 0 1 mM, and a V_{max} value of 100 µM/min for the hydrolysis of *p*NPB.

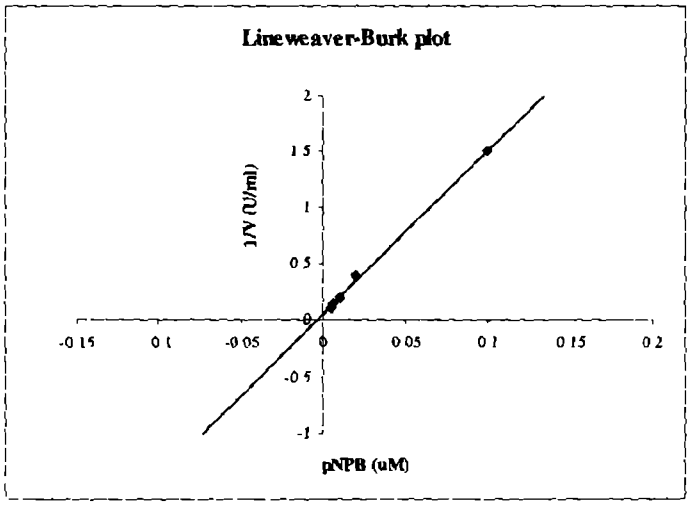


Fig. 4.32 Lineweaver – Burk plot of purified lipase of *B.smithii*

4.7.2.6 Positional Specificity.

Thin layer chromatography revealed that the *B.smithii* lipase is nonspecific in nature as it is able to hydrolyse all possible bonds in the Trioleine there by causing the complete breakdown of the Trioleine molecule (Fig:4.33).

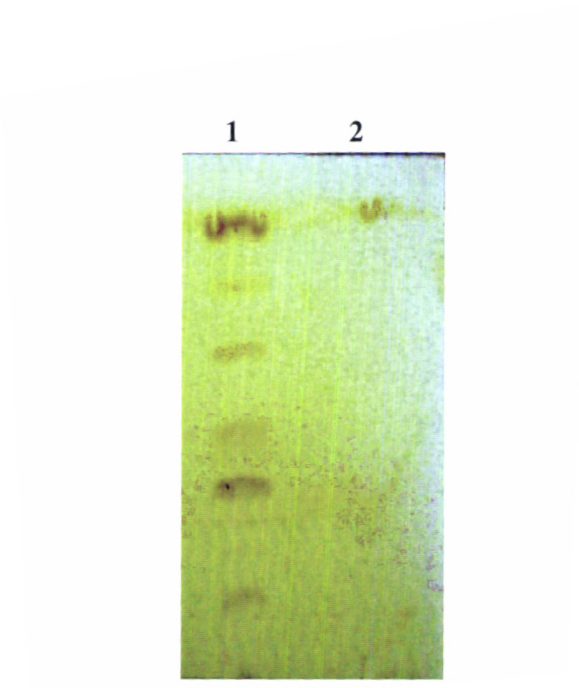


Fig: 4.33 TLC showing the positional specificity of *B.smithii* lipase

Lane 1 Trioleine hydrolyzed by *B.smithii* lipase
Lane 2 Trioleine

4.7.2.7 Effect of metal ions and compounds

The effect of various metal ions on lipase activity was investigated and the results are shown in Table 4.12. The result reveals that all ions

tested were found to enhance lipase activity except Cu^{2+} which showed a inhibitory effect on the lipase activity at higher concentrations (10mM) but showed a positive effect at 1mM and 5mM concentrations. Fe^{2+} ions showed complete inhibition of the lipase activity at concentrations above 1mM but at 1mM it strongly supported lipase activity.

Table 4.12 Effect of various metal ions on lipase activity of *B.smithii* BTMS 11

Metal Ions		Residual activity (%)		
		1 mM	5 mM	10mM
Calcium chloride	**Ca^{2+}**	127	95	93
Cupric sulphate	**Cu^{2+}**	52	70	0
Cobalt chloride	**Co^{2+}**	126	180	161
Ferric chloride	**Fe^{2+}**	233	0	0
Manganese chloride	**Mn^{2+}**	95	98	105
Magnesium sulphate	**Mg^{2+}**	97	103	111
Potassium chloride	**K^{+}**	101	99	68
Sodium chloride	**Na^{+}**	90	95	105
Zinc sulphate	**Zn^{2+}**	103	102	100

4.7.2.8 Effect of different chemical agents.

Significant enzyme inhibition was observed with group-specific potential inhibitors, such as SDS, DEPC, EDTA and PMSF at all the concentrations tested (Table 4.13) β-mercaptoethanol showed a positive effect on the lipase activity

Table 4.13 Effect of various chemical agents on lipase activity of *B.smithii*BTMS11

Chemical agents	Concentration (mM)	Residual activity (%)
	50 mM	0
	100 mM	0
SDS	150 mM	0
	1 mM	68
	5 mM	190
β-mercaptoethanol	10 mM	289
	1mM	0
	5 mM	0
PMSF	10 mM	0
	1mM	0
	5 mM	0
DEPC	10mM	0
	1 mM	18
	5 mM	4.7
EDTA	10 mM	0.47
	0.1	0
H_2O_2	0.5	0
	1.0	0

4.7.2.9 Effect of organic solvents

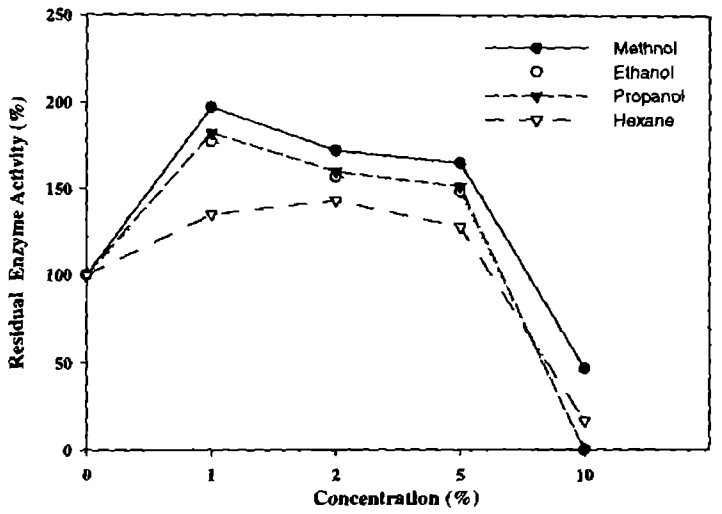


Fig:4.34 Effect of Organic solvents on Lipase activity.

Organic solvents showed a significant effect on the lipase activity. All the solvents tested drastically increased the lipase activity at the concentrations up to 1% level and the activity decreased gradually later upto 5% and steeply at 10% of all the organic solvents tested.

4.7.2.10 Substrate Specificity

B. smithii lipase showed a wide range of substrate specificity and showed activity with all the p-Nitrophenyl substrates tested. As shown in the Fig: 4.35, maximum activity was observed with p-Nitrophenyl Butyrate (C_4) followed by p-Nitrophenyl Caprylate(C_8) and p-Nitrophenyl Acetate(C_2) Comparatively less activity was observed for p-Nitrophenyl Laurate (C_{12}) and p-Nitrophenyl Palmitate(C_{16}). Hence the lipase shows more preference for short to medium chain fattyacids (C_4 to C_8) than to the long chain fattyacids (Fig. 4.35)

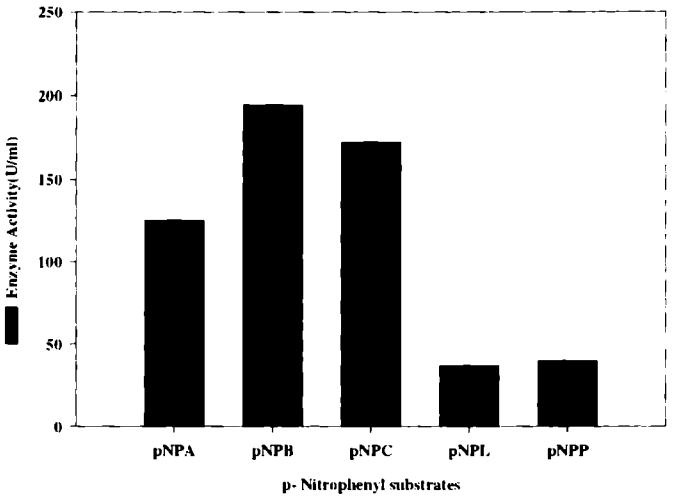


Fig:4.35 Substrate specificity of *B.smithii* Lipase

4.8 Application Studies.

4.8.1. Evaluation of enzyme for use in detergent formulations

In order to determine the suitability of enzyme for use in detergents, its compatibility was studied in various detergent ingredients including surfactants and commercial detergents, by directly incorporating these into the assay mixture.

4.8.1.1 Effect of surfactants on lipase activity.

Stability of B.smithii lipase in the presence of different surfactants was checked and the results indicate that all the surfactants checked showed a significant increase in lipase activity. Maximum activity was depicted by Gum Arabic followed by PEG 6000, Tween 80 and Triton X 100. The stability demonstrated by lipase in the presence of surfactants, marks its potential in detergent formulations.

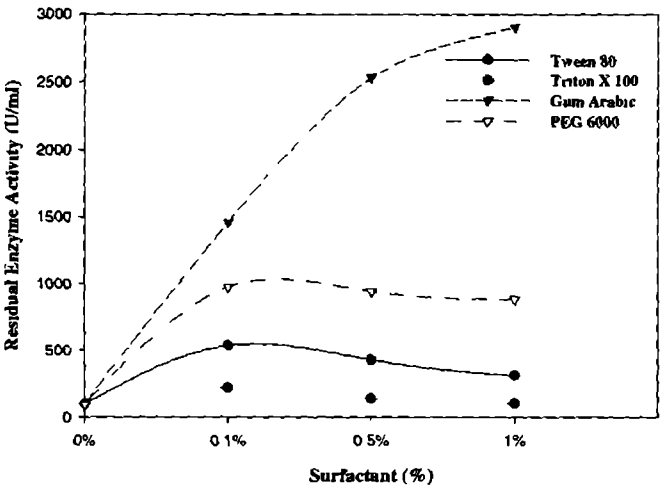


Fig: 4.36 Effect of Surfactants on Lipase Activity

4.8.1.2 Commercial detergent compatibility of enzyme

Commercial detergent compatibility of the enzyme was tested by incubating lipase in detergent solutions for a period of 3 hrs and evaluating the residual enzyme activity. Results depicted in Fig. 4 37, indicated that in all the detergents, more than 90% of activity was retained even after 3hrs of incubation All the detergents tested were observed to be 100% compatible to the lipase since there was no decrease in enzyme activity. This result can be explained from the pH value of each solution (Table 4.14), where all the detergents, have an alkaline pH Since the *Bacillus smithii* lipase has been observed to have alkaline pH range of activity, it is likely to retain its stability in the presence of the detergents

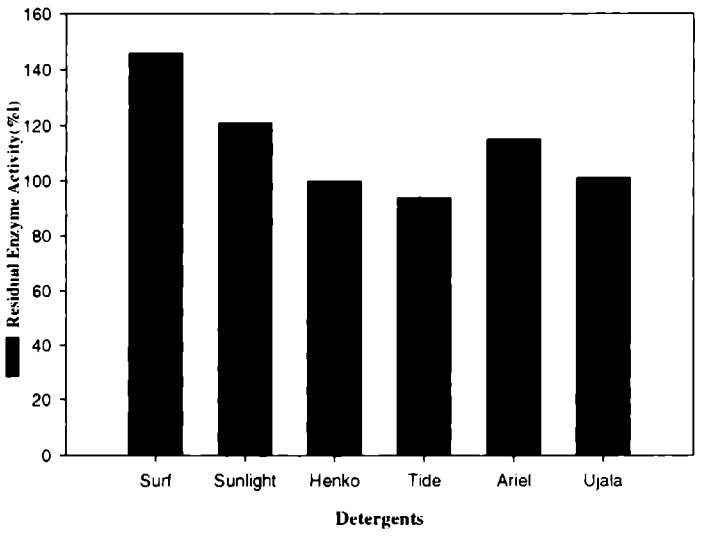


Fig:4.36 Stability of *B.smithii* lipase in the presence of commercial detergents

Table 4.14: pH of different commercial detergent solutions

Detergent	pH of 7 mg/ml solution
Ujala washing powder	9.79
Surf excel	9.70
Ariel compact	9.92
Henko stain champion	10.12
Tide	9.89
Sunlight extra bright with colour lock	10.00

4.8.1.3 Wash performance studies

Wash performance analysis of lipase was studied on white cotton cloth piece stained with oily stain. Visual examination of the stained cloth pieces subjected to wash treatment exhibited the effectiveness of *B.smithii* lipase in removal of stains (Fig. 4.38).

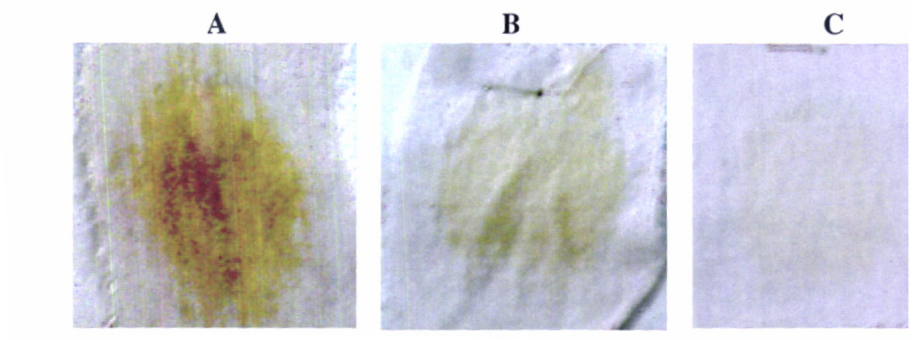


Fig: 4.38 Wash performance study of lipase

A-Cotton cloth stained with oily stain
B- Stained cloth washed with commercial detergent.
C-Stained cloth washed with commercial detergent + *B.smithii* lipase

4.8.2 Esterification study of lipase of *B.smithii*

The ability of *Bacillus smithii* to catalyse a wide variety of esterification reactions was examined. It was observed that *B. smithii* can very effectively carry out Methyl-ester synthesis between fatty acids of varying carbon chain lengths and Methnol. However, in all the esterification reactions, the lipase showed a high esterification capability with all the fattyacids used. The lipase showed preference for medium-chain to long chain fatty acids (C8: 0 to C18: 0) which were etherified at higher conversion rates (70%) in

comparison to very short chain fatty acids though there was fairly good percentage of conversion with short chain fattyacid (52%)(Table:4.15) .

Table 4.15: B.smithii lipase-mediated esterification of fatty acids with Methanol

Fattyacids	Ester Conversion %
Butyric	52%
Caprylic	80%
Lauric	79%
Mystric	78%
Palmitic	73%
Stearic	70%

Chapter 5

DISCUSSION

5.1 Screening, Isolation and Identification of a potential Lipase producing bacteria

Most commercially useful lipases are of microbial origin. Hence the screening program for the isolation of potential lipase producing microorganisms with improved properties has become inevitable. 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 actinomyces. A simple and reliable method for detecting lipase activity in microorganisms has been described by Sierra (1957). This method uses the surfactant Tween 80 in a solid medium to identify a lipolytic activity. The formation of opaque zones around the colonies is an indication of lipase production by the organisms. Also, screening of lipase producers on agar plates is frequently done by using tributyrin as a substrate (Cardenas *et al.*, 2001) and clear zones around the colonies indicate production of lipase. Screening systems making use of chromogenic substrates have also been described (Yeoh *et al.*, 1986). Wang *et al.* (1995) used plates of a modified Rhodamine B agar to screen lipase activity in a large number of microorganisms. Other versions of this method have been reported (Kouker and Jaeger, 1987; Hou, 1994).

In the present study, screening conducted in Tween 80 agar, Tributyrine agar followed by Rhodamine-Olive oil agar plates showed significant results. A single potential lipase producing strain was selected after intensive screening shedule. This isolate was identified as *Bacillus smithii* by MTCC, IMTECH, Chandigarh. 16s ribotyping indicated that the strain has close similarity with the already existing 16s sequences of the *Bacillus smithii* in the NCBI database thereby confirming its identity as *B.smithii.*

The genus *Bacillus* constitutes a diverse group of rod-shaped, Gram-positive aerobic or facultative bacteria that are characterized by their ability to produce robust endospores in response to adverse environmental conditions (Slepecky, 1992). These bacteria are ubiquitous in nature, and are relatively easy to isolate from a wide variety of sources including soil and water. *Bacillus* species of industrial importance are vastly applied in the production of several biological products (Schmidt, 2004). These species are important organisms for both fundamental research and industrial applications. Bacilli currently account for 60% of the commercially available proteins synthesized on an economical scale (Bron *et al.*,2004). Bacilli are well-known high-level producers of a variety of extracellular proteins (Yasuhiko *et al.*, 2000). Their capacity to produce and secrete large quantities (20-25 g/l)of extracellular proteins has placed them amongst the most important industrial protein producers (Schallmey *et al.*, 2004). These organisms continue to be dominant bacterial workhorses in microbial fermentations and have been extensively applied in the production of useful biochemicals, antibiotics, insecticides and industrial enzymes. Majority of these proteins are homologous proteins that are naturally

secreted into the growth medium, such as alkaline proteases and amylases (Quax,2003).

Bacillus species are also reported as prominent lipase produces. Till today Six extracellular lipases and two esterases have been described so far to occur in *Bacillus* species. This includes *B.subtilis, B.thermocatenulatus, B.stearothermophillus, B.thermoleovorans, B.licheniformis, B.circulans* and *B.coagulans* (Eggert, *et al.*, 2000.; Dartois *et al.*, 1992). But so far no lipase has been reported to be produced by *B.smithii.* Again there are no reports of production and characterization of a lipase by a bacillus species of marine origin.

5.3 Process Optimization.

Lipase production is influenced by the type and concentration of carbon and nitrogen sources, as well as culture pH and incubation temperature (Walter ., 2005). Previous work on the physiology of lipase production showed that the mechanisms regulating biosynthesis vary widely in different microorganisms (Sharma , 2001).

In the present work, twelve different oils were used as a sole lipid substrate to study the effect of these substrates on the growth and lipase production by *B.smithii.* No other source of organic carbon or nitrogen source was supplied in the media. This was checked with the interest to study the ability of the organism to utilize lipid component as the sole source of carbon. As a result it was observed that the organism is highly potential in utilising all of the provided lipid substrate as a carbon source for its growth and metabolism and showed remarkable production of lipase. This reveals that lipid sources acts as an inducer for lipase production. Lipid substrates have been reported as suitable inducers on microbial lipase production

(Rathi, *et al*, 2001). But out of all the substrate tested the organism showed maximum lipase production with Gingelly oil (42.23U/ml).This was followed by Coconut oil (37.31U/ml), Sunflower oil (31.89U/ml) and Olive oil (26.89 U/ml). In spite of considerable growth with the rest of the oils tested, there was no remarkable lipase production. Hence Gingelly oil was selected as a suitable substrate for lipase production by *B smithii*. Plant oils containing oleic acid and lenoleic acid such as Gingelly oil had been reported as a suitable substrate for the biosynthesis of lipase in *Candida sp* 99-125 (Tanwei Tan *et al* ., 2003). In general, lipase production in microorganisms is enhanced varying not only the lipid source but also its concentration (Maia *et al*, 2001).

Various concentrations of Gingelly oil (0.5-3%) were checked in order to determine the influence of this variable on lipase production by *B. smithii*. This revealed that lipase production increased with increasing oil concentration but after a certain limit there was a steep decrease in the lipase secretion. This may be attributed to the feed back inhibition of fattyacids produced on the media. Similar decrease in lipase production with the increasing substrate concentration has been reported by many workers (Tehreema *et al*., 200; Marta *et al*, 2004). In general, lipase production in microorganisms is enhanced varying not only the lipid source but also its concentration (Maia *et al*., 2001). Though the highest lipase production was observed with 2.5% of gingelly oil (22.54 U/ml), the lipase activity recorded for 1.5% (21.48U/ml) and 2% (21.11U/ml) of gingelly oil was observed to have a very minor difference. Therefore taking into the economy of the production, 1.5% of Gingelly oil was taken as optimum for maximal lipase production. As Coconut oil was also seen as a potential substrate for lipase production, a combination of lipid

substrates i.e Coconut oil and Gingelly oil in various concentration ratios (ranging 1:1, 1:2, 2:1 ratios of Gingelly oil and Coconut oil) were checked for its effect on lipase production. The two substrates were added together into the media right from the beginning of the fermentation, thereby giving the organism the possibility to use both the substrates sequentially or simultaneously depending on its growth and metabolism. But mixed lipid substrates did not improve the earlier results of using individual substrates as sole carbon sources for production, rather a steep fall in the lipase activity was observed. Though the biomass production was observed in all the cases, no significant lipase production was recorded. The lipase production was observed as 8.56 U/ml (G+C, 1:1), 9.58 U/ml (G+C, 2:1), 9.953U/ml (G+C, 1:2) respectively. Lipase activity obtained for the combinations of lipid substrates were very less when compared to the individual substrates. This pinpoints a possible competing effect of different carbon sources. A similar result reported earlier (Dalmau *et al.*, 2001) suggested a possible competing effect of some soluble carbon source or a close relation between the extracellular lipase activity production and fattyacid consumption behind such behaviour.

Bacillus smithii BTMS 11 was subjected to temperature optimisation studies in Minimal media with Gingelly oil. The organism was incubated at temperatures ranging from room temperatures to 50°C for 48 hours. It was noted that the organism showed growth and lipase production with all the temperatures studied. Maximum lipase production was observed at (RT 28±2) room temperature (24.39 U/ml). This was followed by 35°C (23.56 U/ml) and 40°C (17.87 U/ml). There was lipase production even at 45°C (9.07 U/ml) and 50°C (8.66 U/ml) but was comparatively very low. The ability of the organism to grow and produce enzyme at room temperature is an

added advantage considering the production economy. Though there was considerable production but lipase activity showed gradual decrease with increasing temperature. Further studies were therefore undertaken at RT.

The selected bacterial strain *B.smithii* BTMS11 was checked for its growth and lipase production at various pH ranging from pH 2.0 to pH 11.0 in Minimal media containing gingelly oil (1.5%). Studies showed that the organism was able to grow in all the pH ranges. It was interesting to note that inspite of considerable growth in the acidic pH studied up to pH 5, there was no lipase production. Lipase activity was observed to increase exponentially from pH 6.0 (8.06 U/ml) and gradually reached the peak value at pH 8.0 (41.11 U/ml) followed by pH 9.0 (34.12 U/ml) and pH 10.0 (28.1 U/ml) then showed decrease in activity with increasing pH. It was noted that the organism showed good pH tolerance and lipase production over a pH range from pH 6.0 to 11.0 and hence the influence of pH on lipase degradation can be excluded. Hence it was inferred that the organism is highly potent to produce lipase at the alkaline pH ranges. Maximum lipase activity was observed at pH 8.0 and therefore pH 8.0 was taken as optimum for further studies. In general most of the lipases are produced at neutral or alkaline pH (Sztajer *et al.*, 1988). It has been reported that at the optimal pH, the conformation of this lipase is suitable to absorb to the interface of oil/water, helping open the lid that blocks the active site of the lipase to lower the activation energy of hydrolysis (Jaeger *et al.*, 1994).

Different sugars and polysaccharides were used as an additional carbon source along with the lipid substrates supplied to check the effect of these carbon sources on the lipase production. Ten different

carbon sources which were used for the study were Glucose, Fructose, Sucrose, Maltose, Lactose, Xylose, Mannose, Glycerol, Mannitol and Starch. On the basis of the enzyme activity, it was concluded that, good growth but no remarkable lipase production was observed with the addition of various sugars as additional carbon sources showed no remarkable increase in lipase production, except with glucose (42.59 U/ml) where there was a noticeable lipase production compared to the control without any additional carbon source (32.65 U/ml). The organism showed good growth with all the sugars tested. It appears that when glucose is added as an additional carbon source, glucose is prior to the lipid carbon source for initial growth and when the glucose is exhausted, lipid source is used thereby depicting a sequential consumption of carbon source. Marta *et al* (2004) reported increase in lipase production by *Candida rugosa* with the addition of glucose to the media. This is contradictory to the findings of Dalmau *et al* (2000) where addition of glucose along with the lipid substrates repressed the lipase production by *Candida rugosa*. Study of the various higher concentrations of glucose with gingelly oil showed increased lipase production with increasing sugar concentration up to 0.5% (53.93 U/ml) and steeply decreased at higher levels. It has been shown that glucose is essential for production of lipase by *P. fragii* (Akhtar *et al.*, 1980). Lipase production is generally stimulated by the addition of glucose to the production medium in case of many fungi as well such as *Mucor hiemalis* (Alford *et al*, 1980) and *Aspergillus wentii* (Chander *et al.*, 1980). No lipase production was observed with maltose as an additional carbon source. Sugars in low concentration always supported good biomass production indicating the initial consumption of sugars for the growth and metabolism followed by the utilization

of more complex form of carbon source like lipids when the sugars are completely exhausted form the media

Nitrogen sources, including organic nitrogen and inorganic nitrogen sources play an important role in the synthesis of enzymes. Because inorganic nitrogen sources can be used quickly, while organic nitrogen sources can supply many cell growth factors and amino acids, which are needed for cell metabolism and enzyme synthesis. Therefore, both organic sources and inorganic sources are used in lipase fermentation studies (Tianwei Tan *et al*, 2004). Different nitrogen sources were used as an additional nitrogen source along with the lipid substrates supplied to check the effect of these nitrogen sources on the lipase production. The additional nitrogen sources were added to the production media at the rate of 2g/l.Twelve different nitrogen sources which were used for the study. Both organic and inorganic were used for the study.

Addition of various nitrogen substrates as additional nitrogen sources showed maximal increase in growth and lipase production with all the nitrogen sources checked. Among the organic nitrogen sources checked, maximum lipase activity was observed with 0.2% soybean meal (194.90 U/ml) followed by peptone (143.75 U/ml).Tehreema *et al* (2002) also reported the potential of Soybean meal in increasing lipase production by *Rhizopus oligosporus* (Tehreema *et al*, 2002). Defatted soybeanmeal was found to be an optimal organic nitrogen source for lipase production by *Penicillium camembertii Thom* PG-3. (Tianwei Tan *et al*, 2004) Different higher concentrations of soybean meal with gingelly were also checked for its effect on the lipase production. It was observed that increasing concentration of soybean

meal depleted lipase production. So 0.2% of soybean meal was taken as optimum for further studies.

Calcium ions are reported to have profound effect on the lipase production. Hence this respect, production media with gingelly oil as lipid substrate containing different molar concentration of $CaCl_2$ $.2H_2O$ in the media (0.1mM-5.0 mM) was checked for lipase activity. Maximum lipase production was recorded with 0.1 mM concentration of Ca^{2+} (190.51 U/ml). Increasing molar concentrations of calcium ions showed an inverse relationship with both the lipase production and the growth profile of the organism. Similar observation was similar to the earlier finding of Akhtar *et al.* (1980) where Ca^{2+} at lower concentrations was stimulatory to lipase production by *Mucor hiemalis* while at higher concentrations it was inhibitory. However, it is contradictory to the report of Kokusho *et al.*(1982) where Ca^{2+} and Mg^{2+} both were inhibitory to lipase production in *Alcaligenes* spp. strain no. 679.0. Higher concentrations of calcium ions have been reported to support higher lipase production by *Aspergillus terreus* (Ruchi *et al*, 1999).0.1mM of Ca^{2+} was found optimum for the lipase production by *B.smithii* with Gingelly oil as substrate.

Effect of Magnesium ions was also checked for its effect on the lipase production. For this production media with gingelly oil as lipid substrate and containing different molar concentration of $MgSO_4.7H_2O$ (1mM, 2 mM(C) ,3 mM, 4 mM, 5 mM, 10 mM, and 50 mM) was checked for lipase activity. Mg^{2+} ions showed a positive effect on the lipase production and supported enhanced the enzyme production. Mg^{2+} has been reported as potential ingredient in the lipase production media by many workers (Tan *et al*, 2003).Maximum lipase activity was observed at 2mM (Control) of

Mg^{2+} ion concentration. Increasing molar concentrations of magnesium ions showed an inverse relationship with both the lipase production and the growth profile of the organism. 2mM of $MgSO_4.7H_2O$ was found optimum for the lipase production with Gingelly oil as substrate.

Effect of additional NaCl concentrations on the lipase production was checked by adding different concentrations of NaCl namely to the production media with already optimized parameters. The organism showed good tolerance for the increasing concentrations of the NaCl. There was good growth with all the concentrations of the NaCl checked but lipase production was maximum at 0.5% of NaCl (223.84 U/ml) and then showed a steady decrease with the increasing concentrations of NaCl. This is an added advantage of the marine organism that it can tolerate a high range of NaCl concentration and hence the lipase enzyme produced by this high NaCl tolerant organism is likely to support wash conditions in hard water. Salt tolerance has been reported to enhance the stability of haemophilic enzymes (Reeve *et al.*, 1994).

Different concentrations of the inoculum were added to the production media to study the effect of inoculum concentration on the bacterial lipase production. It was observed that lipase production showed steady increase with increasing inoculum concentration and reached a peak value at 3% inoculum (230.09 U/ml). The lipase production at 4% (220.37 U/ml) and 5% (223.84 U/ml) of inoculum concentration remained close to the activity at 3% of inoculum concentration and then showed depletion in activity with increasing inoculum concentration after 6% (90.04 U/ml). This depletion may be attributed to the over populated culture and fixed amount of nutrients

in the media. The organism starts liberating proteolytic enzymes enhancing self consumption (Sarda *et al.*, 1998)

Time course experiment when conducted for 72 hours under optimized condition revealed that the enzyme production started at 6th hour of incubation and reached a peak (1108.33U/ml, 694.37mg/ml) at 24 hour. The lipase was secreted in to the media at the late exponential phase to the early stationary phase as reported earlier for other organism (Rathi *et al.*, 2001; Bernard *et al*, 2000; Dalmau *et al*, 2000).There was considerable lipase production even up to 36 hours but a steep decrease in activity was recorded at 42 hour and then there was no further increase in the enzyme activity. Growth dependent lipase production was observed also in the case of *B.smithii* which can be linked to the sugar utilization strategy of the organism. At higher inoculum levels, glucose was utilized for faster growth without a lag phase and when the available glucose gets exhausted, a shift towards utilization of the available lipid source for growth and metabolism. However, at lower inoculum densities, due to slower growth leads to delayed utilizations of sugar, and lipase production was delayed.

The lipase activity was found to be synchronous with the growth as reported by many authors (Bernard *et al.*, 2000; Ivana *et al.*, 1998). Hence it unveils that the organism is capable of utilizing lipid source for its growth and metabolism right from the beginning of its growth. In case of *B.smithii*, lipase started to appear in the production media from the 6th hour of growth (12.50 U/ml; 9.09 mg/ml) and increased steadily up to 12th hour (37.50 U/ml; 15.51 mg/ ml) and 18th hour (236.11 U/ml; 177.93) with the simultaneous increase of biomass. Maximum lipase production was achieved at 24th hour (1108.33 U/ml; 694.37 mg/ml). Though there was fairly good production of

lipase even at 30th (539.35 U/ml; 343.42 mg/ml) and 36th (375.93 U/ml; 287.47mg/ml) hours, the production appeared to decrease at 42nd (34.72 U/ml; 37.87 mg/ ml) and 48th hours (27.31 U/ml; 22.25 mg/ml) of production. After that only traces of lipase production was recorded up to 72nd hour. So the protease production was observed towards the stationary phase. In *Bacillus* species generally proteinases and lipases are normally observed to be synthesised in the late exponential and early stationary phases of growth, before sporulation (Priest., 1977).Different types of proteinases are commonly produced in the same medium (Chopra *et al.*, 1985).The synthesis of extracellular serine proteinase has been associated with *Bacillus* sporulation (Strongin *et al.*,1982). It was interisting to note that protease production was observed only after 48th hour when there was a very minimal quantity of lipase in the media. This is an added advantage to the lipase production by *B.smithii* that, no proteases appeared in the media up to 48th hours when lipase was secreted into the media. Hence there occurs no possibility of degradation of lipase produced at its peak level of production. But the secretion of protease enzyme though less was observed to have a close relationship with the depleting lipase production and reducing biomass in this case of *B.smithii.* The decrease in lipase activity suggests proteolytic lipase degradation as reported earlier (Fatima *et al.*, 1999).

5.3 Statistical Media Optimization-Plackett & Burman Experimental Design

Evaluation of factors affecting lipase productivity is an important strategy for the bioprocess development. It has been reported that the growth conditions, the composition of the medium and the physiological age of the culture affect the lipase production (Lopes *et*

al, 1999).Experimental design techniques present a more balanced alternative to the one factor at a time approach to fermentation improvement (Myers and Montgomery, 1995). Stastical approach for the optimization of the media effectively tackles the problem which involves specific design of experiments which minimizes the error in determining the effect of parameters and the results are achieved in the economical manner (Krishnan *et al*, 1998). Plakett –Burman design comprises of two level screening designs and can be constructed on the basis of factorial design (Plakett and Burman, 1946). This design allows the shortlisting of a small number of ingredients for further optimization and allows obtaining unbiased estimates of linear effects of all factors with maximum accuracy for a given number of observations, the accuracy being the same for all effects.

From the results of Plakett and Burman design it is inferred that among the eleven parameters screened, the Gingelly Oil, pH, $CaCl_2$ and Incubation time has a positive influence on lipase production, whereas the temperature, soybean meal, Ammonium sulphate, concentrations of NaCl, $MgSO_4$ and inoculum concentration shows a negative effect on lipase production by bacillus smithii under submerged fermentation.

Statistical testing carried out using Fisher's test for analysis of variance (ANOVA) revealed that the model is statistically significant.The Model *F*-value of 17.49, and values of prob > *F* (<0.05) indicated that the model terms are significant. The test model was statistically significant with a confidence levels above 95%. The quality of fit of the polynomial model equation was expressed by the coefficient of determination (R^2), which equaled 0.9091, indicating that 90.92 % of the variability in the response could be explained by the model. The R^2 value closer to 1.0 predicts the strength of the

response . The purpose of statistical analysis is to determine the experimental factors, which generate signals that are large in comparison to the noise. Adequate precision measures signal to noise ratio. An adequate precision of 13.867 for lipase production was recorded. The predicted R^2 of 0.7327 was in reasonable agreement with the adjusted R^2 of 0.8847. This indicated a good agreement between the experimental and predicted values for lipase production. The value of the adjusted determination coefficient (Adj. $R^2 = 0.8571$) was also very high to advocate for a high significance of the model. These results indicated that the response equation provided a suitable model for the Plackett–Burman design experiment.

5.4 Enzyme Purification & Characterization

The lipase of *Bacillus smithii* in the harvested cell-free broth was optimally precipitated at 60-80% (w/v) ammonium sulfate saturation. The precipitates were reconstituted in a minimum volume of 0.01M Tris buffer, pH 8.0, and were extensively dialyzed against the same buffer. The anion exchange chromatography of lipase on DEAE–cellulose column resulted in single prominent peak. Lipase enzyme purified by ammonium sulphate fractionation and ion exchange chromatography yielded 1.6 and 4.33 fold of purification respectively.

Purified fraction subjected to reductive and non-reductive SDS-PAGE analysis yielded a single band, which confirms the homogeneity and purity of the enzyme. Non reductive SDS-PAGE showed a single band thereby unveiling the monomeric protein nature of the enzyme. The SDS-PAGE performed under reducing conditions also yielded a single band, revealing the single polypeptide nature of the enzyme. Molecular weight estimated for the *B.smithii* lipase by

comparing the electrophoretic mobility of marker protein in SDS-PAGE yielded a value of 45kDa. The lipolytic activity of the purified enzyme protein, confirmed by zymogram analysis using Methylumbelliferyl butyrate as substrate indicated a single type of extracellular lipase Bacterial lipases are reported to span a molecular weight range of 19 to 60 kDa (Iwai *et al*, 1980).The lipases from the two mesophilic strains *B. subtilis* and *B. pumilus* stand apart because they are the smallest true lipases known (approx. 20 kDa) *B. thermocatenulatus* and *B. stearothermophilus* produce lipases with similar properties with a molecular mass is approx. 45 kDa and they display maximal activity at approx. pH 9.0 and 65 °C (Kim *et al.*, 1998., Schmidt., 1996).

Amongst *Bacillus* spp., *B. coagulans* NCIMB 9365 (Molinari *et al.*, 1996) and *B. circulans* (Kademi *et al.*, 2000) possess intracellular lipases while a recently reported thermophilic *B.coagulans* BTS-3 isolate possessed an extra-cellular (31kDa) alkalophilic lipase (Kumar *et al.*, 2005). In contrast, lipase with very high molecular weight (112kDa) has been reported from a mesophilic *Bacillus* sp. (Dosanjh *et al.*, 2002). Thermostable lipases from many *Bacillus* species have been found to possess a molecular mass of 43–45 kDa (Lee *et al.*, 2001; Nawani *et al*, 2000).

The protein nature of enzymes means that pH will affect the ionization state of the amino acids which dictate the primary and secondary structure of the enzyme and hence, controls its overall activity. A change in pH will have a progressive effect on the structure of the protein and the enzyme activity (Fullbrook, 1996).Though the lipase produced by *B.smithii* showed pH optimum of 8.0. The enzyme was active over a pH range of pH 7.0-pH 10.0. The enzyme was completely inactive in the acidic pH range (pH 3.0-pH6.0). It has been reported that at the optimal pH, the conformation

of this lipase is suitable to absorb to the interface of oil/water, helping open the lid that blocks the active site of the lipase to lower the activation energy of hydrolysis (Jaeger *et al*, 1994.).Generally, bacterial lipases have neutral (Dharmsthiti and Luchai 1999; Lee et al. 1999) or alkaline pH optima (Kanwar and Goswami 2002; Sunna *et al.* 2002), with the exception of *P. fluorescens* SIK W1 lipase, which has an acidic optimum at pH 4.8 (Andersson *et al.*, 1979). Lipases from *Bacillus stearothermophilus* SB-1, *B. atrophaeus* SB-2 and *B. licheniformis* SB-3 are active over a broad pH range (pH 3–12) (Bradoo *et al.*, 1999). These results are in accordance with the earlier reports of alkaline lipases of *Bacillus thermocatenulatus*, *Bacillus subtilis*, *Staphylococcus hyicus* and *Pseudomonas fragi* which showed optimal activity in the pH range of 8–10 (Schmidt *et al.*, 1996). Several workers found optimal lipase activity in the highly alkaline pH range (8.5–10.0) from *Bacillus* strain A30-1 (ATCC 53841) (Wang *et al*, 1995) and *B. stearothermophilus* L1 (Kim *et al.*, 1998).Extreme pH conditions alter the structure of the surface of the enzymes modifying the interaction between active site and substrate. Because of that, under strong acidic and alkaline conditions, enzymes are denatured and as a consequence, their activity is totally or partially lost. It could be inferred that the way in which the activity of the enzyme is altered (when they are exposed to different pH for a period of time) is derived from their primary and tertiary structure rather than been due to an adaptation to the pH of the habitat where the producing strains strive (Sreeja, 2005)

Bacterial lipases possess stability over a wide range, from pH 4 to pH 11 (Dong *et al.* 1999). Lipase of *B.smithii* was inferred stable over a wide range of pH from 6-10 However; maximal residual enzyme activity was recorded in the sample incubated in the buffer having pH

8. Nevertheless, when compared to other levels of pH tested, the difference was only marginal. Lipase retained residual activity between pH 6.0 and pH 9.0, but showed a steady decrease at pH 10.0. Stability of the enzyme at the alkaline pH range makes them a potential candidate for application in industrial process to be carried in the alkaline range especially in the detergent industry.

Bacterial lipases generally have temperature optima in the range 30–60°C (Dharmsthiti *et al.*, 1998; Litthauer *et al.*, 2002).The present study revealed that the lipase enzyme of *B.smithii* was active over a broad range of incubation temperature (30°C-70°C) with maximal activity at 50°C. Temperature above 60°C led to a sharp decline in enzyme activity. The lipase activity showed a linear increase along with increase in temperature during 30°C – 50°C. Relative activity profile of the enzyme shows that the enzyme retained 60% of its activity at 60°C and about 30% of its activity at 70°C and 80°C respectively. Data documented on relative activity at different temperatures indicated clearly that the enzyme has the ability to tolerate higher temperatures of 50°C -60°C for maximal activity. It would be extremely favorable for industrial or diagnostic use if *Bacillus* sp. lipase could function up to 60^{0}C (Sugihara *et al.*, 1991). Hence the lipase of *B.smithii* has the potential to be used in the various biotechnological industries.

Temperature stability studies conducted shows the lipase of *B. smithii* BTMS11 possessed that the enzyme retained 60% of its residual activity even after 12 hrs at 30°C to 80^{0}C. Whereas the optimal temperature of 50°C degrees the enzyme retained 58% of activity after 12 hrs of incubation. The enzyme could retain its 50% of activity even at 70-80°C after12 hrs. On the other hand, lipase from *B. thermolevorans* ID-1, exhibited only 50% residual activity at 60

and 70 °C after 60 and 30 min, respectively (Lee *et al*, 1999) and *B. thermocatenulatus* (Wang *et al.*, 1995) lipase which showed 48.5% activity at 60 °C after 30 min of incubation. Temperature optimum of 45 °C or higher for lipase activity has been reported in many *Bacillus* species (Lee *et al.*, 2001.; Jinwal *et al.*, 2003).Most of the industrial processes involving enzymes operate at temperatures above 50°C, the importance of thermostable enzymes is of great significance.(Sharma *et al.*, 2000).The stability of thermophilic proteins is intrinsic and resides in their primary structure. Thermostabilization of proteins is achieved through optimization of intramolecular interactions, packing densities, internalization of hydrophobic residues and surface exposure of hydrophilic residues (Jaenicke *et al.*, 1990). Any approach for stabilizing an enzyme, that is for preventing or at least slowing down, its inactivation may be directed either towards displacing the N→U equilibrium (i.e. by stabilizing the native state relative to the unfolded) or towards preventing the essentially irreversible process which occurs after U is produced (Gray, 1995). Hence the stability at higher temperatures highlights the potential of *B.smithii* lipase candidature in the industrial sector.

In many cases, lipases appear to obey Michaelis–Menten kinetics (Guit *et al.*, 1991). Michaelis–Menten kinetics are characterized by two parameters, K_m and v_{max}. The latter is the maximum rate of reaction and Km is a measure of the affinity of an enzyme for a particular substrate. A low Km value represents a high affinity. The Km values of the enzyme range widely, but for most industrially relevant enzymes, Km ranges between $10^{-1}$and 10^{-5}M (Fullbrook, 1996). The reaction kinetics of the purified enzyme of *B.smithii* was determined from Lineweaver-Burk plots with *p*NPB under standard assay conditions (30 min, pH 7.0, 37^0C). The enzyme had an

apparent K_m value of 0.1 mM, and a V_{max} value of 100μM for the hydrolysis of *p*NPB. The Lineweaver–Burk plots were linear that indicating that hydrolysis of various *p*-nitrophenyl esters by *B. smithii* lipase followed Michaelis–Menten kinetics.

In order to determine the positional specificities of both enzymes, thin layer chromatography (TLC) were performed. The chromatogram was compared with the results obtained and that published by Dong-Woo Lee *et al* (2001) for BTID-A *Bacillus* lipase. The major hydrolysis products 1,2- and 1,3-diolein and 2-monoolein were detected as a main product by *B.smithii* lipase. These results indicated that *B.smithii* is likely to be positional nonspecific one. The positional specificities of enzymes suggest that this enzyme probably act synergistically to hydrolyze extracellular lipids to free fatty acids and glycerols. 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 *et al.*, 1985). Usually nonspecific lipases are considered as the potential candidate for the detergent industry and Novo group has reported a highly alkaline, positionally non-specific lipase from a strain of *Streptomyces* sp. that was useful in laundry and dish-washing detergents, industrial cleaners.

The presence of metal-ions has been previously reported to influence the hydrolytic activities of microbial lipases both of bacterial as well as fungal origin. Two possible mechanisms of ion action were suggested by Lee and Rhee (1993): (1) direct inhibition of the catalytic site, like many other enzymes; (2) specific for lipases formation of complexes between metal ions and ionized fatty acids, changing their solubility and behavior at the interface. Metal ions and salts are of importance in thermostable enzymes from thermophilic

organisms. Stabilization of enzymes by metal ions at high temperatures is by metal ion complexation, which is a process with a favorable entropy factor (Gray, 1995). This is because water previously bound to the hydrated metal ion in solution is liberated when the metal ion becomes bound to the protein. Thus, the process is favored at high temperatures. A number of enzymes require the presence of metal ions, such as calcium ions, for the maintenance of their stable and active structures. These ions are bound strongly to specific binding sites on the surface of the molecules. The binding sites are usually constructed from negatively charged carboxylate side-chain groups of aspartyl and glutamyl residues, brought together by folding of the polypeptide chain (Gray, 1995). Dissociation constants for the binding are low (of the order of 10^{-3} to 10^{-6} M) in the case of $E+Ca^{2}\leftrightarrow E - Ca^{2+}$, representing very strong binding and also emphasizing that the effects took place at low calcium ion concentrations. As can be seen from the above equation, the situation is similar to that of enzyme-substrate complexes. The polypeptide chain is 'cross linked' by the metal ions bridge and the enzyme–calcium ion complex should, therefore, be more rigid and hence more stable (Gray, 1995). The bridging by metal ions in this way is compared to that brought about by disulfide formation. In the absence of calcium ions, the binding site would represent a high local concentration of negative charges. The tendency of these groups to move apart to reduce the repulsive electrostatic interactions would contribute to the relative instability of the folded protein (Sharma *et al*, 2002).

The effect of various cations at a concentration of 1mM, 5mM and 10mM on the activities of *B.smithii* lipase was assessed. The result reveals that all ions tested were found to enhance lipase activity

except Cu^{2+} which showed a inhibitory effect on the lipase activity at higher concentrations but showed a positive effect at 1mM and 5mM concentrations. Fe^{2+} ions showed complete inhibition of the lipase activity at concentrations above 1mM but at 1mM it strongly supported lipase activity. The lipase activity of another thermophillic *B. coagulans* BTS-3 isolate enhanced by K^{+}, Fe^{3+}, and Mg^{2+} ions while Co^{2+}, Mn^{2+}, and Zn^{2+} inhibited the enzyme activity (kumar *et al.*, 2005). Na+ ions have been reported to be essential for lipase activity of *Pseudomonas pseudoalkaligenes* (Lin *et al.*, 1995) as observed in the case of *B.smithii* BTMS11.

The lipolytic activity of cell-bound lipase from a bacterial isolate SJ-15 was mildly enhanced by Cu^{2+} and Li^{+}, mildly decreased by Ba^{2+}, Fe^{3+}, Co^{2+}, and Cd^{2+}, inhibited to the extent of 20–30% by Na+, Ca^{2+}, Mg^{2+}, and K^{+}, and strongly inhibited (75%) by Zn^{2+} ions (Gupta *et al.*, 2004).Another thermophillic *B. coagulans* BTS-3 isolate lipase activity was reported to be inhibited by Al^{3+}, Co^{2+}, Mn^{2+},and Zn^{2+} while K^{+}, Fe^{3+}, Hg^{2+}, and Mg^{2+} ions enhanced the enzyme activity (Kumar et al, 2005). It appeared that the lipase of *B.coagulans* MTCC-6375 as well as other isolate *B. coagulans*BTS-3 are rich in sulfur-containing amino acids (with –S–S– or –SH groups) that interacted with the Hg^{2+} ions (Kanwar *et al.*, 2006).*B. stearothermophilus* MC 7 lipase was inhibited by divalent ions of heavy metals, entirely by Cu2+ and strongly by Fe^{2+} and Zn^{2+} (Kambourova *et al.*, 2003) Like most other lipases, the enhanced catalytic activity of *B.smithii* lipase in the presence of Ca^{2+} is generally accepted as a result of forming of insoluble Ca-salts of fatty acids released in the hydrolysis and thus product inhibition is avoided.

Effect of various chemical agents on lipase activity was checked and the results shows that *B.simithii* lipase was completely inhibited by SDS, PMSF and DEPC at all the concentrations studied. Inhibition by PMSF, suggests that *B.simithii* is a serine lipase having a serine residue at its active site. So as to determine the presence of a thiol group in the enzyme, the enzyme was incubated with β-mercaptoethanol at the concentration of 1, 5 and 10mM. The enzyme activity was not influenced by β -mercaptoethanol. In contrast, *B.simithii* activity was enhanced by β-mercaptoethanol. This observation indicate that thiol group was not present or not critical for the catalytic function. Similar results were found in some lipases (Dharmsthiti *et al.*,1999.;Lee *et al.*, 1999.; Lotrakul *et al.*, 1997). This feature could be applicable for the synthesis of chiral compounds in nonaqueous solvents (Zaks *et al.*, 1998). EDTA treatment strongly inhibited the enzyme with the increasing concentrations confirming that the enzymes require metal ions for their activity. Similar result was depicted by *B.thermolevorans* ID-1(Dong-Woo Lee *et al*, 2001). Unlike most other bacterial lipases, the *B. smithii* BTMS11 lipase is a metallo-enzyme that is inhibited by EDTA as well as PMSF. This indicates that *B. smithii* BTMS11 lipase also possesses a triad of three amino acids at its catalytic site just like many other lipases (Winkler *et al*, 1990). A purified lipase from *B.coagulans* MTCC 6375 was also reported to be inhibited by EDTA and PMSF.1 mM SDS had a strong inhibitory effect on lipase activity. In accordance to our results, Nawani *et al.* (1998) also found a total loss of activity in the presence of SDS.

Various organic solvents were tested for their effect on lipase activity. A marked stimulation of the enzyme activity was observed upon adding organic solvents to the assay mixture. An increased residual

lipase activity was observed with the increasing percentage concentration of all the organic solvents tested namely methanol, ethanol, propanol, and hexane up to 5.0% (v/v). Methanol and Hexane showed 165% and 128% residual activity at 5.0% concentration. But at 10% concentration the activity was reduced to 47% and 17% respectively for methanol and hexane. In contrast 100% inhibition of activity was recorded for propanol and ethanol at 10% concentration. In studies, on the use of lipase for transesterification and synthesis of esters, the reactions have been allowed to occur in media containing water immiscible organic solvents and a small amount of water because the enzymes are less Susceptible to denaturation in such systems (Sugihara *et al*, 1991). The marked stimulating effect of lipase in hexane was because some lipases have a sheath of water molecule tightly bound to the enzyme. This sheath protects the enzyme's hydrophilic surfaces and may allow retention of the native conformation even in the presence of apolar hydrophobic solvents. Since, the lipase from *Bacillus smithii* BTMS 11 was highly stable in n-hexane, the enzyme might be useful for transesterification and ester synthesis

The substrate specificity of the enzyme was studied with *p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl Caprylate(C8), *p*-nitrophenyl laurate (C12) and *p*-nitrophenyl palmitate(C16) .Maximum activity was observed on pNPB followed by pNPC and pNPA respectively, and comparatively less activity was detected on pNPP and pNPL. This result strongly suggests that the lipase of B.smithii BTMS 11 has strong inclination for short to medium chain fattyacids than for the long chain fatty acids.

Application Studies:

Evaluation of enzyme for use in detergent formulations

Detergent compatibility of the *B.smithii* BTMS 11 enzyme was tested with commercial detergents. The results indicated that the enzyme was highly stable in all the detergents tested as it retained more than 90% of activity in al the detergents even after 3hrs of incubation. The enzyme appears to be highly compatible with Surf Excel (146%), followed by Sunlight (121%), Ariel (115%), Ujala (101%), Henko (100%), Tide (94%). Moreover all these detergents have an alkaline pH which compliments the above observation. The lipase activity was dramatically stimulated with the increasing concentration of surfactants namely Triton X-100, Tween 80, Gum Arabic and PEG 600. Nawani *et al.* (1998) and Mingrui *et al* (2007) also found an enhanced activity in the presence of Triton X-100 and Tween 80. The enhanced activity may be either due to the change in the conformation of lipase by surfactants or by change in the interfacial property i.e. increased adsorption of lipase to the interphase. Again the wash performance study highlights the potential of this lipase as a detergent additive.

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Considering the overall properties of different alkaline lipases of microbial origin and the alkaline lipase studied from the isolate from the marine sediment, *Bacillus smithii* BTMS 11 is better as regards to pH and temperature stability, stability in the presence of surfactants, detergent compatibility and wash performance for a potential application in the detergent industry.

Esterification study of lipase of *B.smithii* BTMS 11

It was observed that *B.smithii* BTMS 11 can very effectively carry out Methyl-ester synthesis between fatty acids of varying carbon chain lengths and methanol. However, in all the esterification reactions, the lipase showed a high esterification capability with all the fattyacids used. The lipase showed preference for medium-chain to long chain fatty acids (C8: 0 to C18: 0) which were etherified at higher conversion rates (70%) in comparison to very short chain fatty acids though there was fairly good percentage of conversion with shortchain fattyacid (52%).

Thus, this specificity of the lipase for medium to long chain fatty acids can potentially be exploited for the synthesis of medium long-medium-chain (MLMS) triglycerides via interesterifications in organic media which are low caloric fats (Kazlauskas and Bornscheuer., 1998). Methyl and ethyl esters of long-chain acids have been used to enrich diesel fuels (Vulfson.., 1994). The esters produced from short-chain fatty acids have applications as flavoring agents in food industry (Vulfson., 1994).

Further, esters has extensive uses as emulsifiers, antioxidants and flavour compounds in the food, pharmaceutical, detergent and oleochemical industries (Saxena *et al.,* 1999).Thus, the present study has shown the versatile catalytic potential of *Bacillus smithii* lipase in terms of chemo-, enantio- and regioselectivity and fatty acid specificity. These properties can be exploited to carry out biotransformations of industrially important organic compounds and different types of esters. The efficient one-step enzymatic method has thus been developed for the synthesis of these compounds, which is

not easily feasible via purely chemical means not only in terms of the number of steps involved but also in terms of selectivity.

Chapter 6

SUMMARY AND CONCLUSION

A potential lipase producing strain form the marine sediment was screened among the 280 isolates and was identifies as Bacillus smithii at MTCC, IMTECH, Chandigarh. The identity was further confirmed by 16s ribotyping and the sequence homology showed 90% identity with the reported sequences of *Bacillus smithii* in the GenBank.

Lipase production by the fungus *Bacillus smithii* BTMF 11, isolated from marine sediments under submerged fermentation was evaluated. Various process parameters affecting lipase production under SmF were optimized towards maximal enzyme production. Strategy adopted for the optimisation was to evaluate the effect of various parameters individually on lipase production under SmF and conduct finally a time course experiment under optimised condition.

A concentration of 1.5% gingelly Oil along with 0.2% Soybean meal, 0.5%Glucose, 0.5% additional NaCl, 0.1mM $CaCl_2$, 2mM $MgSO_4$, inoculum concentration of 3% at room temperature and at pH 8.0 supported maximum lipase production. A time course study under the optimized conditions showed maximum protease free lipase production at 24 hours with an activity of 1108.33 U/ml, and Specific activity of 694.37 mg/ml.

The optimization of various bioprocess variables influencing the lipase production by *Bacillus smithii* employing statistical approach under Smf was done using Plakett and Burman experimental design. The results indicated that among the eleven parameters tested, Gingelly oil, pH, $CaCl_2$ concentration and incubation period had a significant effect on the lipase

production. The test model was statistically significant with the confidence level above 90%.

Lipase was purified employing standard protein purification procedures, which included ammonium sulphate fractionation followed by dialysis, ion-exchange chromatography and electrophoresis. It was observed that 60-80% saturation of ammonium sulphate was optimal for the complete precipitation of *Bacillus smithii* lipase. Ion exchange chromatography furnished a single peak with 4.33 fold of purification.

Enzyme protein, which was eluted with 0.5 M NaCl, gave a single band in SDS-PAGE under non-reducing and reducing conditions which endorse the single polypeptide nature of the enzyme. The molecular mass of lipase estimated by electrophoretic mobility showed that the *Bacillus smithii* lipase has an apparent molecular mass of approximately 45kDa.The lipolytic activity of the enzyme protein band was confirmed by zymogram analysis using methylumbelliferyl butyrate as subtrate.

Bacillus smithii lipase has an optimum pH 8.0 with stability over a wide range of pH from 7.0-10.0. Enzyme was active over a broad range of temperature with maximal activity at temperature 50°C and the enzyme retained 58% of activity after 12hrs of incubation at this temperature. Enzyme retained 50% of activity at 70-80^{0}C after 12 hours of incubation.

Enzyme inhibition by the serine lipase inhibitors PMSF suggests that the lipase has a serine residue at its active site like all lipases. Inhibition of the lipase with the EDTA explains its metalloenzyme character and the need for divalent cations for its catalytic activity. This enzyme has highest affinity for the short to medium chain fattyacids (C4 to C8). K_m and V_{max} of the reaction

was calculated as 100μM and 100 U/ml respectively. Lipase of *B.smithii* was observed to be positional nonspecific.

The enzyme showed considerable amount of activity in the presence of all the organic solvents tested with maximum residual activity in the presence of Methanol and Hexane.

The enzyme showed increase in activity with all the ions tested. Higher concentrations of Cu^{2+} (10mM) and Fe^{2+} (5mM and 10mM) showed an inhibitory effect on lipase activity.

Compatibility of the enzyme tested indicates that in all the detergents (Surf, Sunlight, Ariel, Henko, Tide and Ujala), enzyme retained more than 90% of activity even after 3hrs of incubation. Lipase of *Bacillus smithii* showed increase in activity in the presence of surfactants testes. Further, wash performance analysis confirmed the effectiveness of *Bacillus smithii* lipase in stain removal.

Esterification studies with the lipase of *B.smithii* showed that the enzyme can very effectively carry out ester synthesis between fatty acids of varying carbon chain lengths and methanol with high preference for medium to long chain fatty acids showing 70% of esterification. The enzyme showed 52% of esterification with short chain fattyacids.

Conclusion

The results obtained during the course of this study indicate the scope for the utilization of this marine *Bacillus smithii* for extracellular lipase production employing submerged fermentation. High productivity in submerged fermentation using gingelly oil as lipid substrate, activity and stability in alkaline pH and high temperatures, detergents, metal ions, organic solvents

and surfactants highlights the potential of this enzyme for various industrial applications. Further activity in the presence of standard commercial detergents, wash performance studies and estrification activity evidence to its potential for industrial applications. In a nutshell *Bacillus smithii* stands out as a potential marine alkaline lipase producing bacteria which can be exploited for varied industrial applications especially in detergent industry and lipase mediated organic synthesis and esterifications. There is ample scope for further research investigation on the biochemistry of the enzyme, structure elucidation and enzyme engineering towards a wide range of further applications, besides enriching scientific knowledge on marine enzyme.

Chapter 7

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