"SCREENING OF THE GENOMIC GENE BANK FOR IDENTIFICATION AND CLONING OF GENE ENCODING ENANTIO-SPECIFIC LIPASE ENZYME"

M. Sc. THESIS

BY

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"SCREENING OF THE GENOMIC GENE BANK FOR IDENTIFICATION AND CLONING OF GENE ENCODING ENANTIO-SPECIFIC LIPASE ENZYME"

Thesis

Submitted to the
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by

SADIQ MAJEED

IN PARTIAL FULFILLMENT OF THE
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DEGREE OF

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IN
BIOTECHNOLOGY

SEPTEMBER, 2001

DEDICATED

TO MY BELOVED PARENTS

Dr.ABDUL MAJEED MUNSHI & Mrs.HASSINA MAJEED

CERTIFICATE

This is to certify that the thesis entitled "**Screening of the Genomic Gene for Identification and Cloning of Gene encoding enantio-specific Lipase Enz** submitted in partial fulfilment of the requirements tor the degree of "Master of S **(Biotechnology)**" of the Indira Gandhi Krishi Vishwavidyalaya, Raipur, is a record bonafide research work carried **out** by **Shri Sadiq Majeed** under my guidan supervision. The subject of the thesis has been approved by Student's Ac Committee and the Director of Instructions.

Date

CERTIFICATE – I

This is to certify that the thesis entitled "Screening of the Genomic Gene Bank for Identification and Cloning of Gene encoding enantio-specific Lipase Enzyme" submitted in partial fulfilment of the requirements for the degree of "Master of Science (Biotechnology)" of the Indira Gandhi Krishi Vishwavidyalaya, Raipur, is a record of the bonafide research work carried out by Shri Sadiq Majeed under my guidance and supervision. The subject of the thesis has been approved by Student's Advisory Committee and the Director of Instructions.

No part of the thesis has been submitted for any other degree or diploma (certificate awarded etc.) or has been published/ Published part has been fully acknowledged. All the assistance and help received during the course of the investigations has been duly acknowledged by him.

Date

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CERTIFICATE - II

This is to certify that the thesis entitled "**Screening of the Genomic Gene Bank for Identification and Cloning of Gene encoding enantio-specific Lipase Enzyme**" submitted by **Shri. Sadiq Majeed** to the Indira Gandhi Krishi Vishwavidyalaya, Raipur in partial fulfillment of the requirements for the degree **of Master of Science** in the **Department of Biotechnology** has been approved by the Student's Advisory Committee after oral examination in collaboration with the external examiner.

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A CKNO WLEDGEMENT

Research is an evolving concept. Any endeavor, in this regard is challenging as well as exhilarating. It implies the testing of our nerves. It brings to light our patience, vigour and dedication.

Every result arrived at is a modest beginning for a higher goal. My work in the same spirit, is just a step in the ladder. It is a drop in the ocean. No work can be turned as a one-man show. It needs the close cooperation of friends and colleagues and the guidance of experts in the field to achieve something worthwhile and substantial.

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College of Agriculture
Raipur (C.G.) **Sadiq Majeed**

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List of abbreviations and conversion scale

Abbreviation	Expanded Name
μf	Microfaraday
μl	Microliter
ATP	Adenosine triphosphate
BHIG	Brain heart infusion glycerol
bp	Basepair
CCC	Covalently closed circular
CIAP	Calf Intestinal Alkaline Phosphatase
DNA	Deoxyribose nucleic acid
EDTA	Ethylene diamine tetraacetic acid
EMBL	European Molecular Biology Laboratory
GES	Guanidium EDTA sarcosyl
GTE	Glucose tris EDTA
IPTG	Isopropyl-b-D-thiogalactoside
Kb	Kilobase
Kda	Kilodaltons
Kv	Kilowatts
LB	Luria broth
M	Molar
MCS	Multiple cloning site
mM	Millimolar
PCR	Polymerase chain reaction
POP	Performance optimized polymer
Rnase	Ribonuclease
SDS	Sodium dodecyl sulphate
TAE	Tris acetate EDTA
TDNA	Total deoxyribose nucleic acid
TE	Tris EDTA
TSR	Template suppression reagent
X-gal	5-Bromo-4-chloro-3-indolyl-b-d-galactoside

CHAPTER-I

INTRODUCTION

Lipases are produced by a variety of organisms. In eukaryotes, lipases are involved in various stages of lipid metabolism including fat digestion, adsorption. reconstitution and lipoprotein metabolism (Desnuelle 1986). In plants, lipases are found in energy reserve tissues. However, enzymes with industrial potential are usually obtained from microorganisms that produce a wide spectrum of extracellular lipases. Microorganisms producing lipases are widespread and have been found in various natural habitats (Godtfredsen 1990; Sztajer *et al.,* 1988) like industrial vegetable oil plants and dairies, sludges, soil contaminated with oil, rape seeds, compost heap, coal tip. hot springs and old decayed sample of food products.

Lipases are triacylglycerol acylhydrolase (EC 3.1.1.3), capable of catalysing the breakdown of ester bonds of water insoluble substrates, such as natural oils, fats, cholesterol, esters, a large number of drugs and other bioactive molecules or their intermediates. Interfacial activation, a unique property, which makes it possible for the enzyme to perform the lipolytic reaction at the lipid, water interface. The importance of lipases also stems primarily from their ability to preferentially hydrolyse long/short or saturated/unsaturated fatty acyl residues, with a positional specificity of the triacylglycerol molecule. In addition they also remain active in a variety of organic solvents where they can catalyse various transformations (ester synthesis, transesterification, alcoholysis, ester exchange other than hydrolysis).

Chemical synthesis of a product is very tedious, expensive and also leads to synthesis of unwanted side products and thus enzyme mediated reactions are attractive substitute in the chemical industries. The demand of industrial enzymes, particularly of microbial origin, is ever increasing owing to its application in a wide variety of processes and in a large number of fields such as food, dairy, pharmaceutical, detergent, textile and cosmetics. The world market for industrial enzymes has been estimated to be approximately US$600 million. The versatility of lipases makes it an enzyme of immense importance in the industrial market. Lipases thus comprise USS20 million out of the world market of industrial enzymes estimated to be USS600 million.

In the area of detergents, about 1000 tonnes of lipases are sold every year (Godfrey and West 1996). Lipase catalysed transesterification reaction replacing palmitic acid by stearic acid to provide the stearic-oleic and stearic triglyceride with the desired melting point for use in chocolate (Coleman and Macrae 1980). Other applications of increasing interest include use of lipases in removing the pitch from pulp in the paper industry, in flavour development of dairy products, beverages and in synthetic organic chemistry.

The world of bacterial lipases is rapidly expanding. An impressive number of lipase genes have been identified and many lipase proteins were biochemically characterised. The existing three dimensional structure of lipases allow the identification of domains and amino acid residues involved in substrate binding, catalysis and enantiopreference, thereby enabling researchers to tailor lipases for selected applications by using site-directed mutagenesis. Because this approach is

limited to only a few specific cases, the creation of lipases with novel properties by directed evolution constitutes a more general approach. Undoubtedly, there is a steadily increasing demand to identify, characterize and produce lipases for a variety of biotechnological applications, with special emphasis on enantioselective bio-transformants. Therefore, as a first step, standard assay system should be developed, allowing one to test hydrolysis and synthesis reaction catalysed by a given lipase. Furthermore, at least one system that allows for heterologous expression and secretion of different lipases needs to be developed. Finally a data bank should be built comprising sequences of available genes and specific activities and stereoselectivity, and options available to express and produce these lipases. *Bacillus subtilis* is among gram-positive bacteria, the model organism in which a number of genetic tools have been developed and in which extensive genetic analysis is available. In addition, the genetics of the other extra cellular hydrolytic enzymes e.g., proteinases and amylases has been investigated. The present investigation was carried out with the following objectives:

1. To locate the gene encoding enantio-specific lipase enzyme in the selected strain of *Bacillus* species.
2. Design DNA cassettes for enzyme activity.

CHAPTER-II

REVIEW OF LITERATURE

2.1 Lipases

Lipases (triacylglycerol acylhydrolase EC 3.1.1.3), have been widely used in the hydrolysis and transesterification of triglycerides and in the enantioselective synthesis and hydrolysis of a variety of esters (Kotting and Eibl 1994, Wohlfart *el al.,* 1992). In addition to hydrolyzing esters, lipase can catalyze transesterification (Zaks and Klibanov 1984), esterification (Zaks and Klibanov 1985), and aminolysis or oximolysis (Kirchner *et al.,*1985) under anhydrous conditions. Lipolytic reactions occur at the lipid-water interface where the lipolytic substrates usually form equilibrium between monomeric, micellar, and emulsified states (Tilball 1998, Songer 1997). In eukaryotes, lipases are involved in various stages of lipid metabolism (Desnuelle 1986). In plants, lipases are found in energy reserve tissues. However, enzymes with industrial potential are usually obtained from microorganisms, which produce a wide spectrum of extracellular lipases. The importance of lipases stems primarily from their ability to preferentially hydrolyze long/short or saturated/unsaturated fatty acyl residues, but they also exhibit a positional specificity for either the 1 (3) or 1,2 (2,3) positions of a triacylglycerol molecules (Macrae 1983). In addition, they also remain active in a variety of organic solvents where they can catalyze various transformations other than the hydrolytic reactions by which they are defined. The versatility of lipases is therefore being exploited industrially, either to replace existing processes or to produce a variety of different compounds previously not deemed possible.

2.2 **Properties of lipases**

Purified lipases have been characterised for molecular size, isoelectric point, and metal binding capabilities, glycoside phosphorus contents and substrate specificities. Primary structure of several lipases have been determined either from amino acid or nucleic acid sequences. Lipases have been found to be acidic glycoproteins of molecular weight ranging from 14,000 to 86,000 kDa. Several *Pseudomonas* lipases range from 29.000 to 35,000 which are significantly different from those reported for *Pseudomonas fragi* AF03458 (14,600), *P. fluorescence* MC50 (55,000), *S. hyicus* (86,000) and *S. aureus* (70,000) enzyme. (Bozoslu *et al.,* 1984; Kugimiya *el al.,* 1986; Lee and Landolo 1986; van Oort *et al,* 1989 and Gilbert *et al,* 1991). All the bacterial lipases are composed of a single type of subunit which can apparently undergo a variable degree of aggregation. Our knowledge of the structure of lipases and esterases has increased in recent years through the elucidation of many gene sequences and resolution of many crystal structures (Cygler and Schrag 1997). The three dimensional structure of bacterial lipases revealed that they share similar folding pattern not only within themselves but also with other hydrolytic enzymes like haloalkane dehalogenase (Franken *et al,* 1991), acetylcholinesterase (Sussman *et al,* 1988), Dienelacetone hydrolase (Pathak and Ollis 1990) and serine carboxylpeptidase (Liao *et al.,* 1992). Since all of them catalyse hydrolytic reaction, the common folding pattern was named the a/ß hydrolase fold (Ollis *et al,* 1992). Lipases sequenced to date share sequence homologies including a significant region, Gly-X-ser-X-Gly that is conserved in all. The serine residue is suspected to be essential for binding to lipid substrates (Antonian, 1988).

Most of the purified lipases contained between 2 to 15 per cent carbohydrates, the major glycoside residue in all cases being mannose. *G. candidum* lipase contained about 7 % carbohydrate, mainly mannose, and a very small amount of lipid. The lipase had an isoelectric point of pl 4.33 and a molecular weight of 54,000 and contained neither cysteine nor methionine. Optimal activity of *G. candidum* lipase is at 20°C and pH 7.0 and Ser-His-Glu triad forms the catalytic site of the lipase (Schrag *et al.,* 1991). There has been observed a close association of the extracellular lipase from *P. aeruginosa* with lipopolysaccharides (Stuer *el al.,* 1986) although the mechanism of this association is not known.

2.3 Sources of microbial lipases and classification

Lipase positive microorganisms have been shown to be found in various natural habitats (Godtfredsen 1990; Sztajer *et al,* 1988). Microorganisms producing lipases are widespread. Majority of these are mesophillic organisms and include fungi, yeast, bacteria and actinomycetes (Table 2.1). Several reports have appeared on the synthesis of multiple extracellular lipases (isozymes) by fungi, *Viz., C. cylindracea/rugosa*(Rua *et al.,* 1993), *G. candidum* strains (Sidebottobe *et al.,* 1991), *C. antartica* (Heldt-Hansen *el al.,* 1989), *R. Miehei* (Huge-Jenson *et al.,* 1987), *Rhizopus* (Iwai and Tsujisaki, 1974), *Saccharomycopsis lipolytica* (Taylor *et al.,* 1986) and *Penicillium cyclopium* (Iwai *et al,* 1975). The microorganisms involved in lipase production and the families of lipolytic enzymes are detailed in Table 2.1 and 2.2.

Table 2.1: Microorganisms involved in lipase production (Soni and Gupta 1999)

Microorganism	Remarks
I. Fungi	
Rhizopus microporus *R. nodosus* *R. chinensis* *R. delemar* CDBBH313 *R. oryzae* *Aspergillus oryzae* *A. japonicus* *A. awamori* *A. fumigatus* *A. niger* *Pencillium crustosum* *P. cyclopium* *Mucor lipolyticus* *M.javanicus*	Optimum temperature and pH range for their enzyme production varies between 30-45°C and 7.0-8.0 respectively.
II. Yeasts	
Candida cylindracea *C. rugosa* *C. curvata* *C. antartica* *Rhodotorula minuta* *Saccharomyces fagilis* *Rhodotorula pilimonae* *Saccharomyces lipolytica* *Rhodotorula glutinis*	Optimum Lipase production occurs at 30-40°C.
III. Bacteria	
Bacillus megaterium *B. cereus* *Staphylococcus canosus* *S. aureus* *C. licheniformis* *B. subtilis* *B. circulans* *Bacillus stearothermophilus* *P. fluorescens* *Pseudomonas fragi* *Pseudoalcaligenes* *P.stutzeri* *Pseudomonas aeruginosa* *Pseudomonas glumae*	*Bacilli* and *Pseudomonads* are common amongst the bacteria genera capable of producing significant levels of extracellular Lipase.

Table 2.2: Families of lipolytic enzymes.

Family	Sub-Family	Enzyme-Producing Species	Similarity[b] (%)	Properties
I	1	*Pseudomonas aeruginosa*[a]	100	True Lipases
		P.fluorescens	95	
		Vibrio cholerae	57	
		Acinetobacter calcoaceticus	43	
		Pseudomonas fragi	40	
		P. wisconsinensis	39	
		Proteus vulgaris	38	
	2	*Burkholderia glumae"*	35	
		Chromobacterium viscosum[a]	35	
		Burkholderia cepacia[a]	33	
		Pseudomonas luteola	33	
	3	*Pseudomonasfluorescens SIKW1*	14	
		Serratia marcescens	15	
	4	*Bacillus subtilis*	16	
		B.pumilus	13	
	5	*Bacillus stearothermophilus*	15	
		B. thermocatenulatus	14	
		Staphylococcus hyicus	15	Phospholipase
		S.aureus	14	
		S.epidermidis	13	
	6	*Propionibacterium acnes*	14	
		Streptomyces cinnamoneus	14	
II		*Pseudomonas aeruginosa*	100	o.m. bound esterase
		Aeromonas hydrophila	31	Acyltransferase
		Salmonella typhimurium	17	o.m. bound esterase
		Photorhabdus luminescens	17	
		Streptomyces scabies[a]	15	
III		*Streptomyces exfoliatus*[a]	100	Extracellular Lipase
		S.albus	82	
		Moraxella sp.	33	Lipase 1
IV		*Moraxella sp.*	100	Lipase 2
		Archaeoglobus fulgidus	28	Carboxylesterase
		Alicyclobacillus Acidocaldarius	25	
		Pseudomonas sp. B11-1	24	
		Alcaligenes eutrophus	24	
		Escherchia coli	20	Esterase

contd

Family	Sub-Family	Enzyme-Producing Species	Similarity[b] (%)	Properties
V		*Moraxella sp.*	100	Lipase 3
		Psychrobacter immobilis	88	
		Pseudomonas oleovorans	34	PHA-depolymerase
		Haemophilus influenzae	34	Putativeesterase
		Sulfolobus acidocaldarius	25	Esterase
		Acetobacter pasteurianus	15	Esterase
VI		*Pseudomonas fluorescens*[a]	100	Esterases
		Synechocystis sp.	24	
		Spirulina platensis	22	
		Rickettsia prowazekii	16	
		Chlamydia trachomatis	15	
VII		*Arthrobactor oxydans*	100	Carbamate hydrolase
		Bacillus subtilis	48	p-Nitrobenzyl esterase
		Streptomyces coelicolor	45	Putative carboxylesterase
VIII		*Arthrobacter globiformis*	100	Stereoselective esterase
		Streptomyces chrysomallus	43	Cell-bound esterase
		Pseudomonas fluorescence SIK W1	40	Esterase III

[a] *Lipolytic enzymes with known 3-D structure.*
Similarities of aminoacid sequences were determined with the program Megalign (DNA star), with the first member of each family arbitrarily set at 100%.

2.4 Screening of lipase producing microorganisms

Lipase positive microorganisms are found in various natural habitats including industrial vegetable oil plants and dairies, sludges, soil contaminated with oil, rape seeds, compost heap, coal tip, hot springs and old decayed sample of food products (Gowland *et al.,* 1987, Sztajer *el al.,* 1988). Samples from natural habitats are usually enriched with the help of nutrient broth (Gowland *et al.,* 1987) and then screened on agar plates containing salt medium supplemented with yeast extract and emulsified olive oil. Colonies of organism causing clearing of the plate medium are regarded as putative lipase producers. The lipolytic activity of the organisms is also detected on the basis of clearing of the agar

plates containing tributyrin (Arima *et al.*, 1972). Screening systems making use of chromogenic substrates have also been described and found useful for lipase producing fungi (Yeoh *et al.*, 1986). Another simple and reliable method to detect lipase activity in microorganisms has been based on Tweens in the nutrient medium in which the detection of opaque zone around the colony is the proof of lipase activity (Sierra 1957).

2.5 Nutritional requirements and ideal conditions for lipase producing microorganisms

A number of studies have been undertaken to define ideal culture and nutritional requirements for microorganisms to obtain higher yields of lipases. Production of lipase gets substantially enhanced when the type and concentration of carbon and nitrogen source, the initial pH and growth temperature are consecutively optimized.

A salt based medium was used for the production of thermostable lipase from *Bacillus stearothermophilus* (Gowland *et al.*, 1987) containing nutrient broth and Tween-80 (1-1.5%) at 55°C under shaking conditions produced the maximum level of lipase. Of several carbon sources tested, Tween-80 was by far the best growth substrate for lipase production. Tween derivatives such as Tween-20 and Tween-40 are poor substitute for Tween-80. Conditions for optimal production of extracellular lipase from *Pencillium requefortii* S-86 (Marcin *et al.*, 1993) include 2% glucose, initial pH 4.0, growth temperature 27°C and 6 days incubation. Nutritional factors affecting lipase yield by *Rhizopus delemar* include dextrin as the best carbon source (Espinosa *et al.*, 1990). The presence of lipidic source in the growth medium at a level not higher than 2% results in higher enzyme production and Tween-80 exerts a positive effect on enzyme production when used in the range from 0.02% and 2%.

Standardised factors giving optimum yield in *Rhodototula glutinis* (Papaparaskevas *et al.*, 1992) include ammonium phosphate (Nitrogen source), fructose (non-lipidic carbon source), pH 8.0 and temperature 30°C. The parameters affecting the production of extra- and intra-cellular lipase from a thermophilic fungus *Rhizopus oryzae* (Salleh *et al.*, 1993) indicate peptone as the best substrate for extracellular lipase production, but for intra-cellular lipase production other substrates such as tryptone, tryptic digest, polypeptone and corn steep liquor give comparable results. Amongst lipidic substrates, glycerol is the only stimulator of extra-cellular enzyme production, whereas olive oil, triolein and oleic acid have positive effects on intra-cellular enzyme production. Shaking enhances the production of both types of enzymes; 45°C and 37°C are optimum for production of extra- and intra-cellular lipases respectively, while pH 5.0 is optimum for production of both types of enzymes.

The lipase production by five thermophilic fungi, including *Emericella rugulosa*, *Humicola* sp., *Thermomyces lanuginosis*, *Penicillium purpurogenum* and *Chrysosporium sulfureum* (Venkateshwarlu and Reddy, 1993), indicated yeast extract medium to support maximum lipase activity at temperature 45°C by all the fungi. Lipase synthesis occurred in *Aspergillus niger* in a medium without lipids (Pokorny *et al.*, 1994), but for improved production an inducer was needed. The source and concentration of an inducer has no significant effect, starch as an additional carbon source stimulates lipase biosynthesis when used in small amounts.

Butyric acid 1% (v/v) was found to be good inducer. Ota *el al.*, (1966) observed that fatty acid esters such as Tween and Span as well as synthetic glyceride tripalmitin were excellent inducers in the presence of cholesterol for *Candida rugosa*

lipase. A highly hydrophobic lipase from a strain of *Pseudomonas* species was produced in peptone, yeast extract and NaCl medium with 1% olive oil as inducer. The enzyme production reached maximum after 68.5 hr of growth (Kordel *et al.*, 1991).

Gilbert *et al*, (1991) studied the physiological regulation and optimised the production of lipase in *P. aeruginosa* EF2 under Tween 80 limitation. Most fatty acids and their esters act as inducers for the production of lipase.

Most of the reported lipases from the *Pseudomonas* family showed alkaline pH optima with acidic pI values (Svendsen *et al.*, 1995). *P. fluorescens* lipase was stable for thirty minutes at 40°C and pH 7.0; it had more than 80% of the relative activity between pH 5.0 to 11.0 at 37°C for sixty minutes. The lipase appears to be single chain protein containing neither sugar nor lipid. About 20% of the enzyme structure was in a helical configuration.

2.6 Secretory **pathways for lipolytic enzymes**

Lipases are either membrane bound or extracellular. For the extracellular lipases the enzyme has to be translocated through the bacterial membrane to reach their final destination. At present three main secretion pathways have been identified (Salmond and Reeves 1993; Binet *et al.*, 1997) and it appears that lipases can use at least two of them. Type 1 secretory pathway: Lipases from micro-oganisms like *P. flurosecence* (Duong *et al.*, 1994) which lack a typical N-terminal signal peptide are secreted by ATP binding cassette exporter consisting of three different proteins (Binet *et al.*, 1997). In *Serrata marcescens*, the inner membrane protein Lip B containing ABC proteins confers substrate specificity to the system. Lip C as a membrane fusion protein is associated with both the inner and outer membrane and Lip D an outer membrane protein. Type II

secretory pathway: Lipases of both Gram-positive and Gram-negative bacteria possess an N-terminal signal sequence mediating their secretion through the inner membrane by means of Sec translocase. In *E. coli* this is a multisubunit protein complex consisting of the soluble dimeric SecA and a membrane-embedded complex formed by SecY, E, D, G and F (Duong *el al.,* 1997). A similar Sec translocase exists in *Bacillus* species (Pugsley, 1993). Folding of the lipase takes place in periplasm after being secreted through the inner membrane in gram negative bacteria hence producing enzymatically active conformation, and is then transported through the outer membrane. In *P.aeruginosa*, lipase is secreted through such a secretion (Filloux *el al.,* 1998 and Jaeger *el al.,* 1996), encoded by 12 xcp genes organized in two divergently transcribed operons. The XCP proteins are located on both in the inner and outer membrane with XcpQ forming a multimeric pore with a diameter of 95°A (Bitter *et al,* 1998). Recently a similar secretion involved in secretion *of P. alicaligenes* lipases was identified (Gerritse *et al.,* 1998) when additional copies of the secretion were expressed from a cosmid introduced into *P. alicaligenes,* the production of extracellular lipase increased significantly. This effect which has been also observed from *P. aeruginosa* lipase suggests that the number of secretion complexes present in wild type strains is probably low thereby posing restriction on the production of extracellular enzyme.

2.7 **Enantio-specificity of lipases**

The specificity of lipase action on triglycerides can be classified in the following three groups. The first group hydrolyses a specific fatty acid in preference to the others. The second group hydrolyzes the ester bonds located in external positions sn-1 and sn-3 (primary esters) as opposed to those in the internal position sn-2 (secondary ester). The

third group hydrolyses the substrate stereoselectively i.e., the ability to discriminate between two enantiomers in the case of a racemic substrate and between two stereoheterotopic but homomorphic (enantiotopic) groups in the case of prochiral acylglycerols (position sn-1 compared with sn-3). The *S. aureus* lipase has a narrow substrate specificity with no positional specificites. It degrades preferentially short-chain triacylglycerols and acyl esters of both p-nitrophenol and umbelliferone. Extracellular lipase from *P. aeroginosa* shows a marked regiospecificity for the 1,3-oleyl residues of triolein (Gilbert *et al.*, 1991). Many lipases have a high degree of enantioselectivity, which depends on the chemical properties of the substrates, origin of the lipases and experimental conditions used (Rogalska *et al.*, 1993 & 1997, Cygler *et al.*, 1994).

2.8 Molecular cloning, sequence homology and expression of lipases

2.8.1 Molecular cloning

2.8.1.1 Plasmid cloning vectors

Since the early days of molecular cloning, bacterial plasmids have received much more attention because of their usefulness as vectors in recombinant DNA experiments. The design and construction of cloning vectors has now become complex and highly sophisticated area of study, yielding a vast amount of information and greater number of specialised vectors for investigations to use. The plasmids can be replicated and maintained in other hosts with foreign DNA. A plasmid cloning vector is small and present in many copies per cell.

pUC 19 is a part of pUC series related plasmids which contains portions of pBR 322 and M13mp19 and can be amplified with chloramphenicol. pUC19 is a small, high copy number *E.coli* plasmid cloning vector which carries a 54 bp multiple cloning site

polylinker. It has (1) *bla* gene, coding for β-lactamase that confers resistance to ampicillin (source-plasmid pBR 322). It differs from that of pBR 322 by two point mutations; (2) Region of *E.coli* operon *lac* containing CAP protein binding site, promoter P *lac*' lac repressor binding site and 5′ terminal part of the *lac Z* α -gene encoding the N-terminal fragment part of β-galactosidase (source-M13mp18/19).

The map shows enzymes that cut pUC19 DNA once. The co-ordinates refer to the position of first nucleotide in each recognition sequence (Fig.2.1).

DNA prepared from plasmid pKKO was cleaved with the restriction endonucleases Hind III and EcoRI. The fragments *of Pseudomonas fragi* encoding lipase were then subcloned into EcoRI and Hind III sites of pUC9, respectively, and transformed into *E.coli* JM83 (Kugimiya *et al,* 1986*). Lip A* gene encoding an extracellular lipase from *Pseudomonas cepacia* was cloned using pUC19 vector (Jorgensen *et al.,* 1990).

A lipase gene (*lip*) and its activator gene *(act)* on a 2.9Kb BglII-EcoRI fragment from *Pseudomonas* sp. KWI-56 were cloned in *E.coli* using pUC19 as a vector (Iizumi *et al.,* 1991). The partially digested DNA fragments of *Pseudomonas putida* were ligated to pUC19 that had been digested with EcoRI and dephosphorylated with alkaline phosphatase and the resulting mixture was transformed into JM-109 cells (Ozaki *et al.,* 1994).

Some of the other plasmid cloning vectors that were used for microbial lipases are given in Table 2.3.

Fig 2.1 Restriction map of pUC 19 cloning vector

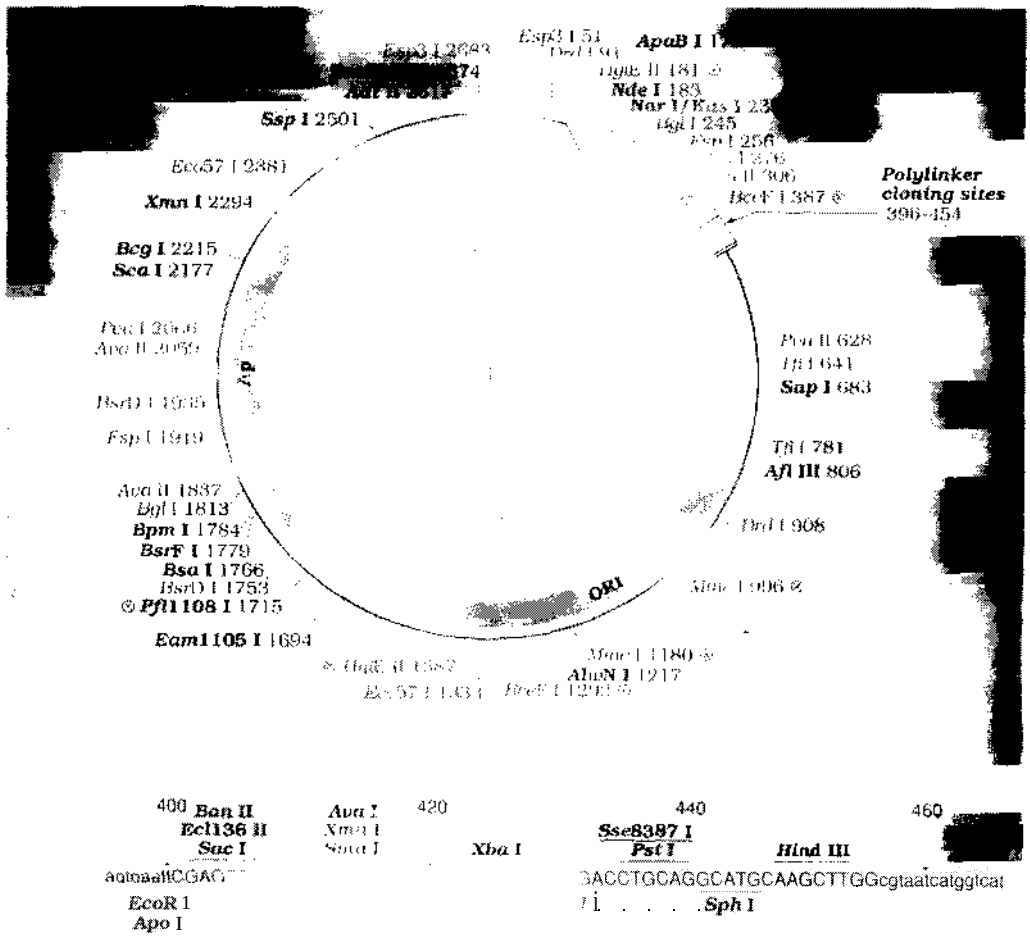


Table 2.3: **Cloning vectors used for microbial lipases**.

S.No	Source	Vector Used	Host
1	*Bacillus subtilis*	PHCB3RLB	*Bacillus. Subitilis* 168AB
2	*Bacillus stearothermophilus*	pUC19 pbluescript	*Escherichia coli* RR1
3	*Bacillussubtilis* WRRL-B558		*E. coli* (JM-109)
4	*Bacillus thennocalenulatus*	pUC 118	*E. coli* (DH5-alpha)
5	*Bacillus* sp. H-257	pACYC 184	*E. coli* PM191
6	*Pseudomonas cepacia*	pCYTEXPl	*E. coli*
7	*Pseudomonas cepacia*	PTV 18CPCmutN	*E. coli* JM-109
8	*Pseudomonas* sp. SD 705	pUC118	*E.* co/i (JM-109)
9	*Pseudomonas fragi*	PUC-RSF Km CCB	*Pseudomonas putida* AC 10
10	*Pseudomonas* PS-21	EMBL3 phage vector	*E. coli* P 2392
11	*Pseudomonas fragi* IFO-12049	pUC9	*E. coli*
12	*Pseudomonas fragi* IFO-3458	pUC9	*E. coli* JM83

2.8.1.2 Microbial transformation

The process of transformation was the first genetic process to be observed in bacteria any yet it remains one of the most remarkable transfer mechanisms. Large DNA fragments (as much as million base pairs in size) are released from a donor cell and diffuse through the culture medium until they encounter other cells. The molecules are then transported into the recipient cells and recombination occurs. Moreover, recent studies have shown that two genetically distinct strains *of Bacillus* can exchange genetic information while growing in sterilised soils.

Much progress has been made towards the realization of the potential advantage *of Bacillus* since 1978 when the first *Bacillus subtilis* cloning experiments were reported (Grcyzan *et al.*, 1978). Several plasmids have been tried as vectors and a number of promoters have been used to express several bacterial and viral genes.

The difficulties encountered when using *Bacillus* as host, have included plasmid instability (both loss of the entire plasmid and genetic rearrangements) and the high protease level of certain strains. Problems associated with plasmid instability may be caused by recombination between homologous and can be sometimes overcome *by* avoidingduplicatedsequencesinconstructs.

Plasmids can be introduced into *Bacillus* by five different methods. transformation of competent cells, transformation by protoplast, transduction using bacteriophage AR9 (Grcyzan *et al.*, 1978), mobilization using the conjugative wide host range plasmids, pAMB1 and by using electrotransformation (electroporation).

2.8.1.3 **Electrotransformation**

Electroporation is rapidly becoming one of the most efficient and versatile techniques for introduction of DNA into the cells. This technique involves the application of brief, high intensity electric fields to cells to reversibly permeabilize the membrane to exogenous molecules (Knight, 1981). Wani (2000) reported an efficient transformation system for selected *Bacillus* strains. Kanamycin substituted plates resolved the identity of the transformants. A major difference between the electroporation of eukaryotes and prokaryotes is the high electrical field required for the latter. DNA introduced into bacteria by electroporation is more vulnerable to restriction nucleases than transferred by conjugation or natural transformation systems.

Electroporation is rapidly becoming the method of choice for transforming gram positive microorganisms which previously were difficult to transform via protoplast/PEG methods (Chassy and Flickinger 1987; Fielder and Writh 1988; Miller *et al.*, 1988; Powell *et al.*, 1988). A simple and easy method for the introduction of plasmid DNA, into

different species of *Bacillus*, involves the suspension in a transformation buffer of nutrient agar grown cells in their late exponential phase and the addition of plasmid DNA. Transformants were obtained at a frequency of about 10 to 10 stable transformants/ug of plasmid DNA.

Kelly *el al.,* (1996) optimised the conditions for electroporation of *Bacillus sphaericus* 2362. increasing transformation efficiency from 10^2 to 10^4 transformants/ug of transforming DNA when the plasmid concentration was kept low. Plasmid DNA isolated from *B.sphaericus* 2362 produced the highest efficiency when electroporated into the same strain.

Wei *et al.,* (1995) reported an optimised method for electroplating foreign plasmid DNA into *Lactobacillus* sp. For electroporation, 1ul of plasmid DNA (0.5μg) was mixed with 50μl of the ice-cold cell suspension in a Gene Pulser disposable cuvette and held on ice for at least 2 min. This mixture was then exposed to a high voltage electric pulse, which was delivered, a strength of 12.5Kv/cm and produced a pulse duration of 7.4-9.4 m/sec for untreated recipient bacteria. Plasmid DNAs ranging from 2.9 to 12.6Kbp were transformed into *Bacillus subtilis* ISW 1214 by electroporation. The transformation efficiency (transformants/μg plasmid DNA) decreased as plasmid DNA size increased. However, 2 X 10^3 transformants /μg DNA were obtained using a plasmid over 12.6 Kbp (Ohse *et al.,* 1997). Comparison of transformation efficiencies with plasmids of various sizes (upto 200Kb) have been studied in several other species of Gram positive and Gram negative bacteria, like *Bacillus cereus* (Belliveau and Trevors 1989), *Bacillus subitilis* (Brigdi *et al.,* 1990). Supercoiled and relaxed circular forms of plasmids upto at least 20Kb transform with the same efficiency.

The concentration of DNA greatly affects the recovery of transformants. With *E. coli*, the frequency of transformation (transformants/survivor) was found to be strictly dependent on DNA concentration. At higher concentration upto 80% of the survivors were transformed (Dower *et al.*, 1988).

2.8.1.4 Screening of the genomic library

Pseudomonas fragi gene bank was constructed in *E.coli* using pUC 9 vector and was screened for lipase activity on tributyrin plates (Aoyama *et al.*, 1988). The genomic DNA of lipase producing *Rhizopus delemar* ATCC 34612 was isolated, digested with MboI, ligated with plasmid pBR 322 (4.3Kb) and transformed into *E.coli* C600 and was verified by colony hybridization using biotinylated fungal DNA as a probe (Haas *el al*, 1990).

Chromosomal DNA of *Pseudomonas fluorescens* was digested with Sau3AI, ligated with plasmid pUC19 DNA and transformed into *E.coli.* About 12,000 recombinant colonies were screened on tributyrin agar plates for esterase activity (Chung *et al.*, 1991). Two lipase related sequences were isolated by screening gene libraries from *Candida cylindracea* (Cc) with synthetic oligonucleotides (Alberghina *et al*, 1991). An extracellular lipase was purified from *Pseudomonas aeruginosa* TE 3285 culture supernatant. A Cynogen bromide (CNBr) digested fragment of the purified enzyme was used to construct a DNA probe for screening of a genomic library of TE 3285 in phage Lambda-EMBL3.

Genomic DNA isolated from *Pseudomonas fluorescens* B52, was partially digested with sau3AI. DNA fragments (2-6Kb) were ligated with plasmid pUC19 and transformed into *Escherchia coli* DH5-alpha. Among 9,000 *E.coli* transformants, 4

colonies formed clear zones on tributyrin agar medium (Tan and Miller, 1992). A DNA sequence encoding a *Pseudomonas* sp.109 lipase is new. *Pseudomonas* sp. was lysed using lysozyme and SDS. and was extracted with phenol. DNA fragments were inserted into a plasmid pUC19 vector, and a gene bank was produced in *Escherchia coll. E.coli* cells containing the new lipase gene were selected using tributyrin culture medium .

Lindahl *et al.,* (1994) constructed the genomic library of *Bacillus subtilis* by ligating *B. subtilis* chromosomal DNA, partially digested with Taq 1. with plasmid pGEM linearised with cla 1. The gene library was replica-plated and screened on peptone agar media containing tributyrin, for lipolytic activity [as a zone of clearing (halo) around a colony].

2.8.2 **Molecular Sequence homology** of Lipases

Determination and comparison of specific activities and substrate specificities of different lipases are absolutely required to investigate the physiological function of lipases as well as to judge their usefulness for biotechnological applications. Fifty three sequences of bacterial lipases and esterases are compared and classified according to conserved sequence motifs and the biological properties (Arpigny and Jaeger 1999) which permits the identification of newly isolated lipolytic enzymes. (Table 2.1).

There are a large number of reports published on the molecular cloning of the lipase gene from different microbial strains. Lipase genes from *S. aureus* were cloned and expressed in *E.coli. B. subtilis* and *S. aureus* (Lee and Landolo 1985). The DNA sequence of lipase gene from *S. hyicus* yielded a preprotein of 641 amino acids with the signal sequence of an N-terminal hydrophilic segment that was followed by a hydrophobic sequence ending in Ala-X-Ala that finally was followed by the sequence for

mature protein. The lipase gene from *S. aureus* also revealed the presence of signal sequence with the classical characteristics of a hydrophilic region followed by hydrophobic amino acids ending in Ala-X-Ala prior to mature protein. Comparison of *S. aureus* and *S. hyicus* lipases revealed 46% homology, a significant amount. The sequence Leu-Val-**Gly**-His-**Ser**-Met-**Gly**-Gly was found in *S. aureus* lipase and it contained the homologous region found in all the lipases sequenced to date.

Many lipase genes (17 Upases) from members of the genus *Pseudomonas* have been cloned and sequenced. Gilbert (1993) classified them into two groups according to amino acids sequence homology. One group consisted of Upases from *Pseudomonas* species strain 109, *P. aeruginosa*, *P. pseudoalcaligenes*, *P. glumae, P. cepacia* and *P. putida* and the other group consisted of lipases from *P. fluorescence* and *P.* sp. strain LS107d2. No similarities between the two lipase groups were found except for well conserved -Gly-X-Ser-X-Gly-sequence which is the active centre of lipase.

The lipase gene from *Proteus vulgaris* K80 was cloned in *E.coli* (Kim *et al.,* 1996). Sequence analysis showed an open reading frame of 861bp coding for a polypeptide of 287 amino acid residues. The deduced amino acid sequence of the lipase was compared with sequence in the SWISS- PROT data bank using the Blitz EMBL program. The most similar enzymes were the lipases from *P. glumae, P. aeruginosa* and *P. fragi* which showed 39.7%, 41.5% and 46.3% homology respectively, indicating that lipase from *Proteus vulgaris* K80 was similar to group I lipase in the respect of molecular size and amino acid sequence similarty. It was also similar to group II lipases of P. *fluorescence, P.* species strain LS107d2 and 5. *carcescens* in that it has no typical signal sequence and does not require an additional protein for production of an active

lipase. Molecular cloning and sequencing of the lipase genes from *S. aureus* PS54, *S. epidermidus* and *S. hyicus* revealed common structural features for this class of enzymes with an N- terminal region of about 260 amino acids acting as a folding catalyst (Gotz *et al.*, 1985 & 1998, and Lee & Landolo, 1986). All three exoplipases are organised as prepro-enzymes and have a molecular mass of approximately 70 kDa. Processing by proteolytic cleavage of the signal and pro-peptides results in the mature lipase forms with molecular masses of 40-46 kDa. The lipase from *S. aureus* NCTC 8530 has also been found to be organised as a prepro-enzyme and the mature part shares approximately 32% identity and 38% similarity on the amino acid level with the other *Staphylococcal* lipases (Nikoleit *et al.*, 1995).

2.8.3 **Expression vectors**

Lipase enzymes found in many different organisms are frequently used during the synthesis of pharmaceutical compounds for racemate resolution to yield a single, desired stereospecific isomer. The use of lipase for large-scale process is expensive and aims to develop an in-house source of lipase. Recombinant protein expression (RPE) has emerged in recent years as a high profile discipline and a research tool for molecular biologist. Today, RPE provides an ever-expanding array of techniques to broaden and enhance research across the spectrum of life sciences.

Plasmid DNA capable of replicating in *E.coli* is formed by inserting a lipase structural gene from a Gram-negative bacterium to *E.coli* vector plasmid. Sources of suitable lipase gene include *Alcaligenes denitrificans*, *A.odorans*, *Pseudomonas putida* while examples of the recombinant plasmid are pYU 1511, pGN 43, pUE 11 and pLP 62. Chung *et al.*, 1991 reported the overexpression of thermostable lipase gene from

Pseudomonas fluorescence in *E. coli* using expression vector plasmid pTTY2 which was constructed by inserting a 1.6 kb EcoRI-PstI fragment (from plasmid pJH92) containing the lipase gene between the tac promoter and the rrnB terminator of plasmid pTTQ19. The amount of lipase produced by the transformed *E. coli* was more than 40% of the total cell protein when induced with isopropyl-beta-D-thiogalactopyranoside. (Johnston and Cino, 1999) reported the cloned lipase gene from *Pseudomonas fluorescens* overexpressed in *Escherchia coli.* The optimized recombinant *E.coli* cells produced approximately 165-fold more lipase than the parent cells. The lipase signal peptide alone was not sufficient for efficient secretion of hybrid protein into the culture medium when gene fusion vectors were constructed from *Slaphylococcal* plasmid pC194 with *E.coli* TEM beta-lactamase gene at various positions of the lipase gene, resulted in fusion proteins with varying lengths of lipase protein. Gene fusion vector, plasmid pTIT2T, constructed using the *Staphylococcus aureus Prolein-A* gene under the control of phage lambda PR promoter (Goetz *et al.,* 1988) resulted in expression of *S. hyicus* gene encoding a lipase with unusual properties in *E. coli* with lipase activity. (Horn *et al.,* 1991) reported the characterization and overexpression of a cloned *Pseudomonas* lipase gene. A 3Kb DNA fragment coding for lipase was transferred to *Pseudomonas* sp. The recombinant strain possessed a lipase activity that was 42-fold higher than the strain without the plasmid. The use of a recombinant *tac*-lipase gene fusion, resulted in 85 fold higher lipase activity in *Pseudomonas* sp. Lipase production in heterologous host using two pseudomonas genes was reported

The cloned bacterial lipase genes in many cases could directly express in the host organism. However, some but not all lipases require a secondary gene for their

activation. Replacement of the *lip*A expression signals by heterologous signal from gram-positive bacteria still gave *lim*A-dependent *lipA* gene expression in the hosts using plasmid pIJ 4642, *Bacillus subtilis* (using plasmid pSJ416) and *Streptomyces lividans* (using plasmid pIJ 702). Lipase gene *lipA*, from a strain of *P.cepacia* have expressed efficiently in *E.coli, B. subtilis* and *Streptomyces lividens* in the presence of a second gene *limA*, which in *P. cepacia* is linked to *lip A* and are located immediately downstream of the lipase genes (Jorgensen *et al.,* 1991) and this might be regulating expression of lipA by effecting correct folding (Hobson *et al.,* 1993). Recent studies of P. *cepacia* {limA (Hobson *et al.,* 1993)}, *Pseudomonas* sp. KW156 {act (Iizumi *et al.,* 1991)}, *P. aeruginosa* {lip B (Hirayama 1993; Yoshikawa *et al.,* 1992 & Wohlfarth *et al.,* 1992)}, *Pseudomonas.* sp. strain 109 {limL (Ihara *et al,* 1995)}, *P. glumae* { lipB (Frenken *et al.,* 1993a)}, *P. cepacia* (Jorgensen *et al.*, 1991) indicated that a secondary gene product is required for the correct folding as well as for the hyperexpression of the lipase gene in heterologous hosts. Rua *et al.,* 1998 reported an efficient expression system for the previously only weakly expressed thermophilic lipase BTL2 gene *(Bacillus thermocatenulatus* lipase 2) was developed for the production of large amounts of lipase in *Escherchia coli* BL321, DH5-alpha and JM 105. The gene was isolated from plasmid pLIP 2 and ligated into the plasmid pCYTEXPl (pT1) expression vector downstream of the temperature-inducible lambda promoter PL .

2.9 Potential application of lipases

The demand of industrial enzymes, particularly of microbial origin, is ever increasing owing to their application in a wide variety of processes. Enzyme-mediated reactions are attractive alternative to tedious and expensive chemical methods. Enzyme

find great use in large number of fields such as food, dairy, pharmaceutical, detergent, textile, and cosmetic industries.

The world market for industrial enzymes has been estimated at approximately US $600 million, with lipases comprising approximately US $ 20million (Arbridge and Pitcher 1989) and comprise a wide variety of different applications (Table 2.3). In the areas of detergents (Godfrey 1996), production of food ingredients, paper industry (Farrell 1997), in flavour development for dairy products and beverages, and in synthetic organic chemistry (Boland *et al.*, 1991; Drauz and Waldman 1995; Faber 1997; Schmid and Verger 1998). Lipolytic enzymes are currently attracting enormous attention because of their biotechnological potential (Godfrey & West 1996; Jaeger & Reetz 1998).

Table 2.4: Application areas of industrial lipases (Godtfredsen, 1990).

Industry	Effect	Product
Dairy food	1) Hydrolysis of milk fat	Flavour agents
	2) Cheese ripening	Cheese
	3) Modification of butter fat	Butter
Bakery food	Flavour improvement and Shelf life prolongation	Bakery products
Beverages	Improved aroma	Beverages
Food dressing	Quality improvement	Mayonnaise, dressings and whippings.
Health Food	Transesterification	Health food
Meat and Fish	Flavour development and fat removal.	Meat and Fish products
Fats and Oils	1) Enantioselectivity	Cocoa butter, margarine, Fatty
	2) Hydrolysis	acids, glycerol, mono and diglycerides.
Chemical	Enantio-selectivity	Chiral building blocks and chemicals.
Pharmaceutical	1) Transesterification	Speciality lipids
	2) Hydrolysis	Digestive aids
Cosmetics	Synthesis	Emulsifiers, moisturing agents.
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper with improved quality
Cleaning	Hydrolysis	Removal of cleaning agents e.g., surfactants

One of the most attractive field of lipase application is the production of monoacyl glycerols, which are most widely used emulsifiers in food, pharmaceuticals and cosmetic industries. Chemically monoglycerides can be synthesized at high temperatures (200-250°C) leading to unwanted side products. Bornscheuer *et al.,* (1994) produced pure monoacyl glycerols using nonspecific *Pseudomonas cepacia* lipase. Lipase catalyzed products have a higher quality and can also be termed natural under some conditions (Armstrong *el al.,* 1989). The major target in the fat and oil industry is the production of new triglycerides types with desirable melting properties using lipase-catalyzed interesterification of readily available triglycerides (Macrae and Hammod 1985). Lipase can be applied for regio selective esterification of polyfunctional alcohol (Seitz 1974). As early as 1913, enzymes were used in detergents by the German chemist Otto rohm, who patented the application of pancreatic enzymes in presoaking composition. The lipases when incorporated into detergents help in removing oil stains from the fabrics. Patent application describing the identification of several lipase suitable for detergent application has been published from *Pseudomonas alcaligenes* by Gist-brocades (Andreoli *et al.,* 1989), the lipase from *Bacillus pumilis* and *P. plantarii* by Solvary (Bycroft and Byng, 1992) and lipase from *P. glumae* by Unilever (Batenburg *et al.,* 1991).

CHAPTER-III

MATERIALS AND METHODS

The study entitled "Screening of the genomic gene bank for identification and cloning of gene encoding enantio-specific lipase enzyme" was carried out at Genetic Engineering Laboratory, Department of Biotechnology, Regional Research Laboratory, Jammu in collaboration with the Department of Biotechnology, Indira Gandhi Agricultural University, Raipur as a joint inter-institutional collaborative research.

3.1 Bacterial strains and media used

Strains of *Bacillus* spp. used in the present investigation for the isolation of lipase gene were RRL-XLRH *(Bacillus licheniformis)*,RRL-BBI *(Bacillus subtilis)* and *Escherchia coli* strains, were used as hosts for transformation. All the bacterial strains and recombinants derived were stored in glycerol (1:1 ratio) broth cultures at -70° C. For regular use, strains were maintained on agar slants.

The different media used for propagating bacterial strains were sterilised by autoclaving at 121° C for 15 minutes.

Table 3.1: Types of Media used in the present investigation.

	Composition of Mcdia/Lt of distilled water and use					
S. No.	Name	Bacto-tryptone	Yeast extract	Sodium chloride	Agar	
1	Luria broth (pH 7.2 to 7.5)	I0g	5g	!0g	15g	Solid media for revival and maintenance of bacterial strains
2		10g	5g	5g		Cryopreservation of genomic library
4	Brain Heart Infusion media	BHI 37g	Glucose 5g	-		Provides stability to the Sphaeroplastsduring electroporation

3.2 Plasmid cloning vectors

In the present investigation. pUC19 plasmid cloning vector was used for preparing the partial genomic library of the strains *of Bacillus* spp. *(Bacillus subtilis* coded as BB-I and *Bacillus licheniformis* as XLRH), and pFLAG-ATS, was used as expression vector.

3.3 Buffers and solutions

The procedure of preparation of solutions and buffers used in the present investigation are detailed elsewhere in Appendix-I. Protocols detailed in ***Molecular Cloning*** by Sambrook *et al* (1989) were followed in the present investigation until and unless stated.

3.4 Qualitative and quantitative estimation of lipase activity in the parental strains and transformants.

3.4.1 Titremetry method (Quantitative estimation)

Substrates were prepared by emulsifying one gram of each triglyceride triacetin, tributyrin and olive oil with 1gram of gum-arabic in 100 ml of distilled water with constant stirring. Ten ml of 1M CaCl2 and 2.5 ml of 1M NaCl was then added to above emulsion and the pH was adjusted to *7.5* with 0.1M NaOH. Fifteen ml of freshly prepared substrate pre-incubated at 37°C for 10 min. on a thermocontrolled pH-stat, was used for assay. After the addition of enzyme solution, the amount of 10mM NaOH required to maintain the pH at 7.5 after every 3 min. was recorded to determine the lipase activity.

$$\text{Lipase activity (units /mg)} = \frac{\text{Vol of 10mM NaOH used}}{\text{3 minutes X Wt. Of cells}}$$

3.4.2 Agar diffusion method (Qualitative estimation)

Five ml of 0.2% emulsion of tributyrin and 0.02% Tween 80 in dH_2O were added to 95 ml of hot solution of Davis agar (1.2%) prepared in 50 mM of Tris.HCl (pH 8.0). Twenty ml of this emulsion was then poured in presterilized plastic petri dishes and was allowed to solidify. Holes of 10mm were bored in the medium and 20µl of lipase solution was loaded per well. The plates were then incubated at 37°C for 1 -2 hr and were observed for the formation of cleared zone which was taken as positive for lipase activity and the diameter of the zone as an qualitative estimate.

3.5 Isolation of chromosomal DNA from *Bacillus* strains

From the overnight growth of 100ml culture, the cells were pelleted by centrifugation at 7,000 rpm for 10 minutes. The supernatant was discarded and 625µl of lysozyme (dissolved in TE pH 8.0) was added to the pellet, and was incubated at 37 C for 30 minutes. To the incubated mixture, 4 ml of GES reagent was added and was vortexed vigorously to lyse the cells. Thereafter, 2.5 ml of ice-cold 7.5M ammonium acetate was added to precipitate the DNA and was allowed to cool on ice for 15 minutes. Five ml of chloroform [octanol or isoamyl alcohol 24:1 (v/v)] was added to the mixture, and vortexed to mix the phases. The mixture was then centrifuged at 15,000 rpm for 15 minutes to separate the phases. The supernatant was collected and was washed twice with chloroform which was then transferred on sterilised petriplate, and one tenth of the volume (of supernatant) of 7.5M ammonium acetate + double volume of chilled ethanol was added to precipitate the DNA. Precipitated DNA was then transferred to a sterilised eppendorf tube, washed with 70% ethanol, air dried and redissolved in 200 µl TE .

3.6 Agarose gel electrophoresis

The plasmid and chromosomal DNA were electrophorased on 0.7 % agarose gel at 80 to 100V and 70 mA for two hours in 1X TAE buffer. Gel was stained with Ethidium Bromide (10 mg/ml) and were then observed on UV-transilluminator.

3.6.1 Elution of DNA (*Bacillus*/Hind III) (1-6Kb) and 2.9Kb fragment of pUC19

Samples of DNA mixed with loading buffer were loaded onto the gel (0.6% low melting agarose gel) and electrophorased at 4° C. The bands of interest were located using a hand-held, long wavelength UV lamp, which minimised the risk of radiation damage to the DNA. The band of interest was then cut out as a slice from agarose using a sharp scalpel and was transferred to a clean, disposable plastic tube. To keep the record of eluted band the gel was photographed.

To the gel containing the band of interest approximately 3-4 volume of TE (pH 8.0) was added. The tubes were then heated for 5 minutes at 68°C to melt the gel, and equal volume of phenol was added. The tubes were vortexed for 15-20 seconds, and the phases were separated by centrifugation. The aqueous phase was extracted with phenol-chloroform once and then with chloroform. DNA was precipitated with ethanol using one tenth volume of 7.5M ammonium acetate and double volume of absolute chilled ethanol. The DNA was then washed with 70% ethanol, air dried under vacuum and dissolved in TE buffer.

3.7 **Plasmid DNA isolation (Alkali lysis method)**

3.7.1 **Maxiprep method**

The cells were harvested from the overnight grown 100ml stationary phase *E. coli* culture at 7,000 rpm for 10 minutes at 4°C. Supernatant was discarded and the pellet was resuspended in 4 ml of solution 1 (Glucose, Tris.HCl and EDTA) by vortexing the cells. To the suspension, 1 ml of lysozyme was added and incubated at room temperature for 5 minutes. From the freshly prepared solution II (2N NaOH, 10% SDS and sterile water in the ratio of 1:1:8) 10 ml was added. and mixed gently by inverting the tube 5-6 times. The tube was kept on ice for 10-15 minutes, and then 7.5 ml of chilled solution III (5M-potassium acetate, glacial acetic acid and sterile distilled water) was added and thoroughly mixed the lysate and incubated on ice for 20 minutes, and centrifuged at 15,000 rpm for 30 minutes at 4°C. The clear supernatant was collected in a new tube and to it, its 0.7% volume of isopropanol was added, and were kept at room temperature for 45 minutes. The precipitated plasmid DNA was centrifuged at 15,000 rpm for 15 minutes. After discarding the supernatant the DNA pellet was washed with 70% ethanol, dried under vacuum and dissolved in 0.5 ml of Tris EDTA.

3.7.2 **Isolation of plasmid DNA from recombinants using ABI prism (Miniprep kit)**

From the overnight grown culture of the recombinant colonies approximately 3ml was centrifuged to pellet the cells. The pellet was resuspended in 150ul resuspension buffer by vortexing. To the suspended cells 150 ul of lysis buffer was added and mixed gently by inverting the tube 5-6 times. After incubating for 3 minutes at room temperature, 150 ul of ice-cold precipitation salt was added

and the tubes inverted 6-8 times to ensure thorough mixing. The mixture was then centrifuged at 14,000 rpm for 5 minutes at room temperature.

The supernatant was poured into a new, sterile. 1.5ml micro centrifuge tube. and the gelatinous pellet was discarded. To the supernatant 600 µl binding buffer was added and was mixed by inverting 5-6 times. The entire solution was poured onto the miniprep column. which was placed inside the collection tube. The miniprep column/collection tube was centrifuged at room temperature at 1,000-3,000 rpm for 30 seconds. thus resulting the plasmid DNA to bound to the column. The column flow through was discarded and 500 µl of wash buffer was added. and was again centrifuged at room temperature at 3,000xg for 30 seconds. Discarding the column flow through 900 ul of ice-cold 1X final wash buffer was added and centrifuged for 30 seconds. Finally column flow through was discarded and miniprep column/collection tube was centrifuged at room temperature at maximum speed for 1 minute to dry the resin.

To elute the plasmid DNA, the miniprep column was placed into a sterile eppendorf tube and 60 ul of TE buffer or sterile water was added to the resin and was incubated for 3 minutes at room temperature. Thereafter, the miniprep column/collection tube was centrifuged at maximum speed for 30 seconds, to elute the plasmid DNA in the collection tube.

3.8 **Transformation**

3.8.1 Preparation of competent cells following glycerol method

Competent cells were prepared of JM-109 in LB incubated at 37°C in a shaking incubator. One ml of fresh overnight culture of JM-109 (*Escherchia coli*)

was inoculated in 100 ml of LB, and was incubated for one hour at 37°C with vigorous shaking. From 50 ml of fresh culture, the cells were harvested by centrifugation at 7,000 rpm for 10 min. The harvested cells were washed twice with sterile water; and subsequently for two times with 10% glycerol by centrifugation. Finally the pellet was dissolved in 1 ml of 10% glycerol and the cell suspension was distributed into aliquots of 50, 100 and 200 ul and stored at -70°C until further use.

3.8.2 Electroporation

The competent cells, which were prepared following glycerol method, were thawed gently at room temperature and were then immediately placed on ice. In a 1.5ml eppendorf tube, 60 ul of cell suspension was mixed with 8 µl of ligation mixture (DNA in low ionic strength buffer) and was left on ice for 1 minute to chill the mixture. The mixture of the cells was transferred to a precold electroporation cuvette, and was placed in a chilled safety chamber slide, and the cuvette was pulsed once at 2.5Kv, 25µf and 200Ω resistance. Cuvette was removed and immediately 1ml of BHIG medium was added to resuspend the cells. The cell suspension was transferred to a polypropylene tube, and was incubated at 37^0C for one hour in shaking incubator set at 225 rpm. Hundred ul of the above mixture was plated on LB media plates incorporated with ampicillin for the selection of recombinants.

3.9 Preparation of partial genomic library

3.9.1 Preparative scale restriction digestion of chromosomal DNA of *Bacillus* strains

Four hundred ul *(Bacillus* TDNA 40ul + 10X Buffer M 40ul + Sterile water 314ul + Hind III 6μl) of reaction mixture was incubated at 37°C for 2½ hr. Restriction digested DNA was subjected to low melting agarose electrophoresis. DNA fragments of 1-6Kb size were removed along with the gel and was stored at -20°C in a centrifuge tube for elution.

3.9.2 Restriction digestion and dephosphorylation of plasmid cloning vector

Hundred uJ (pUC19 DNA uncut 40ul + 10X buffer M 10ul + Sterile water 40μl + Hind III 10ul which was added at the last) of the reaction mixture was incubated at 37°C for 3 hrs. Digested DNA was subjected to low melting agarose gel electrophoresis and the 2.9 Kb fragment of plasmid DNA was cut from the gel, and was stored at -20°C in eppendorf tube. Dephosphorylation of pUC 19 was done using CIAP (Calf intestine alkaline phosphatase). Hundred ul of reaction mixture containing pUC 19 DNA digested with Hind III/CIAP (80 ul) + Alkaline phosphatase buffer (10 μl) + Sterile water *(5* μl) + Alkaline phosphatase enzyme-CIAP (5μl) was incubated at 37°C for 30 minutes. Thereafter, 5ul of the alkaline phosphatase was added again and was incubated at 37°C for another 30 minutes. Heat shock (68°C) for 10 minutes was given to stop the reaction, and the purification was done following phenol-chloroform method.

3.9.3 Ligation of the chromosomal DNA (l-6Kb) fragment with pUC19

For ligation 40 µl of the reaction mixture containing Bacillus DNA/Hind III (8 µl) + pUC 19/Hind III/CIAP (4 µl) + 10X T_4 DNA Ligase buffer (4 µl) + 10X ATP (1 µl) + Sterile water (21 µl) + T_4 DNA Ligase (2 µl) was incubated at 16^0C overnight. The ligated DNA was purified following phenol-chloroform method.

3.9.4 Selection of recombinant clones

Insertion of DNA into the multiple cloning site (MCS) located within the Lac Za inactivates the N-terminal fragment of β-galactosidase and abolishes a-complementation resulting in white bacterial colonies. The recombinant clones were identified by white colour that differed from the untransformed blue colonies. Replica-plating was done to isolate the colonies that were lipase positive on tributyrin plates.

3.9.5 Restriction digestion of plasmid DNA from recombinant clones

Plasmid DNA was isolated from the recombinant clones using the ABI Prism Mini Prep Kit that is detailed elsewhere in materials and methods (section 3.6.2). Restriction digestion of the isolated plasmid DNA was done to look for the presence of DNA fragments inserted from *Bacillus* (1-6Kb).

3.10 Sequencing of the recombinant plasmid

ABI Prism 310 Genetic Analyzer, Macintosh Computer, Genetic analyzer Buffer (Stock concentration 10X and Working concentration 1X), POP (Performance Optimised Polymer-6) 30ml, TSR (Template Suppression Reagent) 4.0ml, M-13 Universal Primer, 3M Sodium acetate (pH 4.8) 24.609g/100ml.

PCR reaction mixture

Recombinant Plasmid DNA	2 µl	
PCR Sequencing mixture	8 µl	= 20
M-13 universal primer	3 µl	
Sterile water	7 l	

Initially the sequencing PCR was performed as per the program given below: -

1. Denaturation temperature	96.0°C	10 seconds
2. Annealing temperature	50.0°C	5 seconds
3. Extension temperature	60.0°C	4 minutes

Go to 1 & repeat 24 cycles

Hold at 4.0°C

3.10.1 Steps for purification and sequencing analysis

About 80µl of sterile water was added to the reaction tube. Then 10µl of 3M sodium acetate followed by 250 µl of absolute alcohol was added to each sample and Vortexed. The samples were incubated on ice for 15 minutes. The samples were centrifuged at room temperature for 30 minutes at 15,000 rpm.

To the pellet 200 ul of 70% ethanol was added and centrifuged for 10 minutes at 15,000 rpm, and was repeated twice. The pellet was air dried and dissolved in 12 ul of TSR. Denaturation of the DNA was performed at 96°C for 3 minutes, and was immediately cooled on ice for 10-15 minutes, and were then transferred to reaction tubes for sequencing.

Sequencing was based on chain termination method and utilises fluorescent rather than radioactive labels. The fluorescent dyes are attached to the sequencing primer / to the dNTPs / or to the terminators, and are incorporated into the DNA during the strand synthesis reaction mediated by a DNA polymerase (e.g., Klenow fragment of DNA polymerase-I, Sequenase or Taq DNA polymerase). The emitted

fluorescence due to laser beam during electrophoresis of the newly generated DNA fragments on a Polyacrylamide gel, was analyzed by computer in the form of a nucleotide sequence. The sequences were then compared using Blast search or by comparing the possible homologies with the different data-banks which are available on www.ncbi.nlm.gov/blast/Blast.cgi website.

3.11 DNA cassettes for **enhanced enzyme activity**

pFLAG-ATS expression vector was used to allow expression, detection and purification of recombinant fusion proteins in *E.coli* strain sure. These vectors offer a choice of periplasmic or cytoplasmic expression with either amino or carboxy terminal tagging. For this purpose, the following reaction was carried out: -

Bacillus (5 Kb insert) Hind III	6 µl	
pFLAG cut Hind III	2 µl	
10X Ligase Buffer	2 µl	
10X ATP	1 µl	= 20 µl
Sterile water	8 µl	
T_4 DNA ligase (1U)	1 µl	

Ligation, preparation of competent cells and electroporation procedures were followed in the similar manner as were used for pUC19 detailed elsewhere in materials and methods (section 3.8.3) except vector used was pFLAG-ATS. Transformants were selected on LB ampicillin and the selected colonies were observed for lipase activity on tributyrin/tween plates.

CHAPTER-IV

RESULTS

Extra-cellular lipases are produced by a variety of microorganisms and the genes for several such lipases have been cloned. Today, lipases from microbial origin stand amongst the most important biocatalysts earning out novel reactions in both aqueous and non-aqueous media. their ability to utilize a wide spectrum of substrates, high stability towards extremes of temperature, pH and organic solvents, chemo-, regio- and enantioselectivity. Enzyme-mediated reactions are attractive alternative to tedious and expensive chemical methods. The demand for industrial enzymes in general, and lipases in particular, is ever increasing owing to their application in a wide variety of processes. In recent years, genetic engineering techniques have been applied in the mass production of these enzymes. *Bacillus* strains are well known for their lipase activities and thus are the potential source for its endogenous lipase genes Thus, the present investigation was carried out with the identification of lipase positive strain from *Bacillus subtilis* and *Bacillus licheniformis*.The identification of positive strains from these isolates prompted us for the formation of genomic library in a suitable host, its screening for lipase activity. The identified cloned fragments were then sequenced and sub-cloned in a designed expression vector for its enhanced expression in *E. coli*.

4.1 Screening of lipase producing *Bacillus strains*

Bacillus strains screened in the present investigation were revived on LB/Agar plates to get a single bacterial colony. Lipolytic activity was identified following titremetry and agar diffusion methods. One strain each from *Bacillus subtilis* and *Bacillus licheniformis* coded as BB-I and XLRH respectively which showed a distinct clearing

zone on LB/Tributyrin/Agar plates and were supposed to possess lipase activity. These were selected for isolation and sequencing of the putative lipase genes (Table 5). The enantio-selectivity of the enzyme produced by the two strains was confirmed by Dr.S.C.Taneja and Dr.S.K.Koul, Department of Organic Chemistry, RRL-Jammu (personal communication).

Table 4.1: *Bacillus* strains used in the present investigation .

S.No	Name of the Strain	Lipolytic activity
1	*Bacillus subtilis* (BB-I)	Lipase positive
2	*Bacillus licheniformis*(XLRH)	Lipase positive

4.2 Isolation of chromosomal and plasmid DNA and their restriction digestion

Chromosomal DNA isolated from *Bacillus* strains (BB-I and XLRH) was purified and was electrophorased on 0.7 % agarose gel, for quantification and to confirm its purity. The isolated chromosomal DNA of Bacillus subtilis (BB-I) was of high molecular weight then that *of Bacillus licheniformis* (XLRH). (Fig.4.1).

The genomic DNA from *bacillus* strains after partial restriction digestion with Hind III were electrophorased on low melting agarose gel (0.6%). Analytical reaction carried out for different time intervals (five, ten, thirty, sixty, ninety and hundred eighty minutes) revealed that restriction digestion for 2^1 hr yielded maximum number of 1-6Kb sized fragments (Fig.4.2). The reaction mixture when increased to 20 folds resulted in a smear, due to the presence of a large number of restricted fragments. Plasmid DNA was subjected to complete restriction digestion for 3 hr with Hind III, which generated two bands on the gel.

Fig. 4.1 Isolation of Genomic and Plasmid DNA

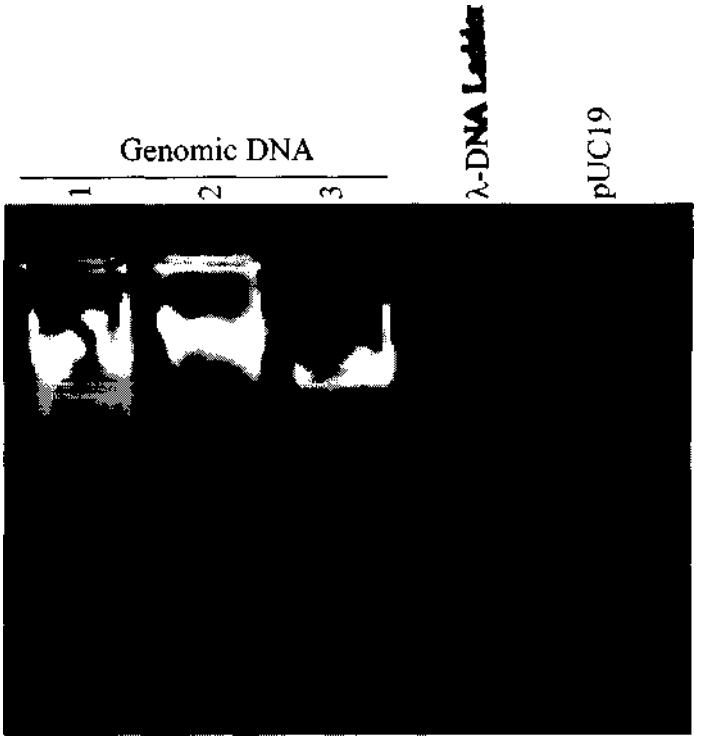
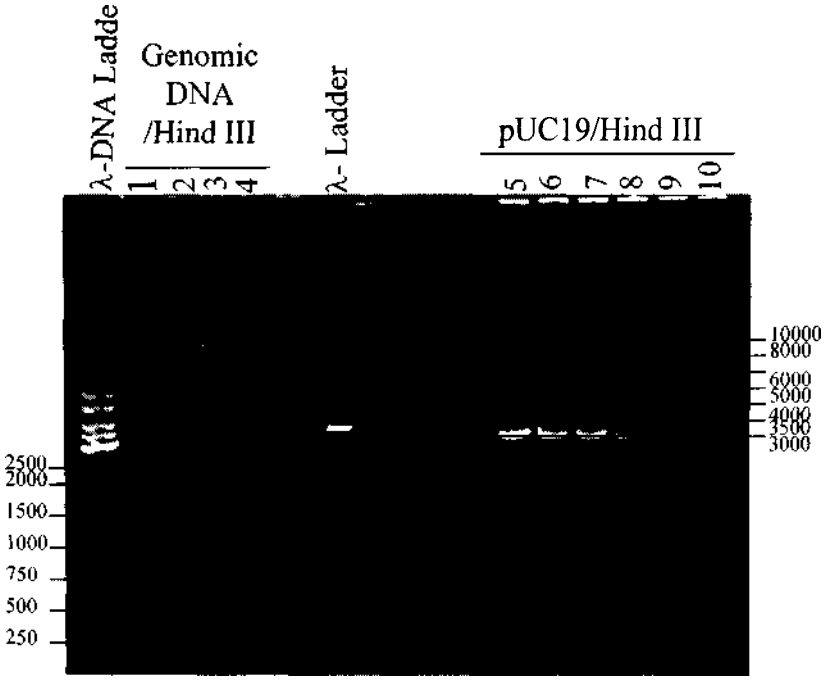


Fig. 4.2 Restriction Digestion of Genomic and Plasmid DNA



4.3 Development of partial genomic library and selection of recombinant clones

The linearized eluted fragment of pUC19 (2.9Kb) was dephosphorylated and was used in ligating mixture for the insertion of eluted chromosomal DNA (1-6Kb) fragments from the two strains *of Bacillus* spp. (BB-I and XLRH) separately. Ligation was optimum when Genomic : Vector DNA were mixed in a ratio of 1:4. The presence of high molecular wt. DNA in the agarose gel (0.7%) confirmed the ligation reaction/the insertion of the genomic (1-6Kb) fragments into the pUC 19 cloning vector.

Competent cells of *E.coli* (JM-109) were prepared following glycerol (10%) method. Care was taken to confirm the purity of the prepared competent cells by plating them on LB/Ampicillin/Agar plates. No growth confirmed the purity of the competent cells. Competent cells were transformed with the plasmid bearing the ligated chromosomal DNA (1-6Kb) fragment derived from *Bacillus subtilis* (BB-I) and *Bacillus licheniformis* (XLRH) separately.

Table 4.2: Transformation efficiency of pUC 19 cloning vector carrying foreign DNA *of Bacillus* spp (BB-I and XLRH) in *E. coli* host

Host strain	Source of Genomic DNA (1-6 Kb) fragments	Time Constant (ms*) for high voltage electric pulse	No.of colonies/200µl of DNA plated out	Efficiency of Competent cells (per µg of DNA)
E. coli	*Bacillus subtilis* BB-I	4.3	2,000	1*10 colonies
E. coli	*Bacillus licheniformis* XLRH	4.5	4,000	$2*10^5$ colonies

Assuming if 100ng of plasmid DNA is transformed in 1ml of culture upon plating 100µl (10ng).
ms* (milliseconds). Time constant during which the cells take up DNA ranged between 4-5ms.

The time constant achieved and the number of transformants obtained varied (Table 4.2). 200µl of electrotransformed mixture was plated on LB/Amp/IPTG/X-gal medium and were incubated at 37°C overnight. The incubated plates showed well distinguished blue and white colonies of *E. coli*. Blue colonies represented the cell containing the intact vector with LacZα–region and thus were able to hydrolyze X-gal, whereas the white colonies represented the recombinant clones as a result of insertional inactivation of the Lac-Z (Fig.4.3).

When the competent cells were transformed with the plasmid, bearing the chromosomal fragment from BB-I, the time constant was observed to be 4.3ms and the number of transformants obtained were less (1 x 10^5 colonies/µg of DNA), to that of when the competent cells were transformed with the plasmid bearing the inserted DNA fragment of XLRH strain, which resulted in a slightly higher time constant of 4.5 ms and double the number of transformants (2 x 10^5 colonies / µg of DNA)

4.4 Screening of the transformants for lipolytic activity

A total of three hundred transformants representing one hundred fifty white colonies each of *Bacillus subtilis* (BB-I) and *Bacillus licheniformis*(XLRH)genomic library were screened on LB/Tributyrin/Agar plates (Table 4.3 and 4.4).

Only two transformants BB-14 containing a chromosomal DNA fragment of *Bacillus subtilis* (BB-I) and XL-16 carrying chromosomal DNA fragment from *Bacillus licheniformis*(XLRH) showed a distinct clearing zone as a result of lipolytic activity on LB/Tributyrin/Tween agar plates, and were thus confirmed as lipase positive.

4.5 Analysis of recombinant plasmid DNA

Plasmid DNA was isolated from BB-14 and XL-16 which showed lipase positive reaction, and also from about 100 recombinant clones showing white colonies but were

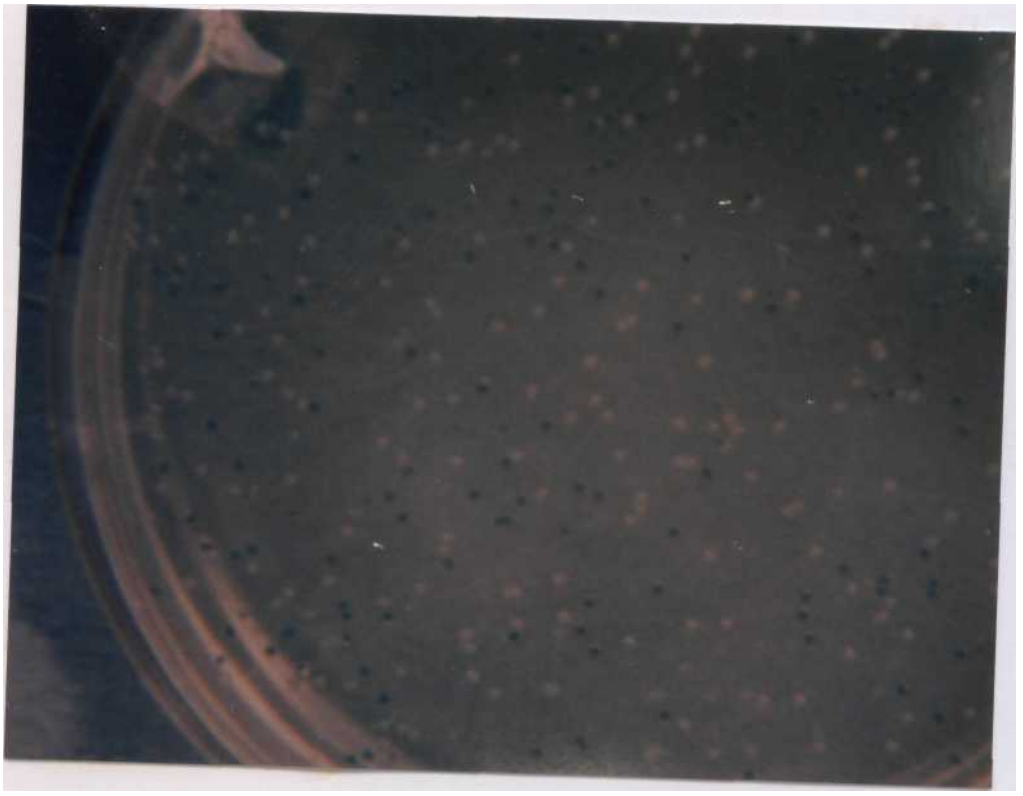


Table 4.3: Observations recorded from screening of BB-I genomic library.

Colony No.	Reaction	Colony No.	Reaction	Colony No.	Reaction
1	- (L)	51	- (L)	101	- (L)
2	- (L)	52	- (L)	102	- (L)
3	- (L)	53	- (L)	103	- (L)
4	- (L)	54	- (L)	104	- (L)
5	- (L)	55	- (L)	105	+ (I)
6	+ (I)	56	- (L)	106	- (L)
7	- (L)	57	- (L)	107	- (L)
8	+ (I)	58	- (L)	108	- (L)
9	- (L)	59	(*)	109	- (L)
10	+ (I)	60	- (L)	110	- (L)
11	- (L)	61	- (L)	111	- (L)
12	- (L)	62	- (L)	112	- (L)
13	- (L)	63	- (L)	113	(*)
14	+ (L)	64	- (L)	114	+ (I)
15	- (L)	65	- (L)	115	- (L)
16	- (L)	66	- (L)	116	+ (I)
17	- (L)	67	- (L)	117	- (L)
18	- (L)	68	- (L)	118	- (L)
19	- (L)	69	- (L)	119	- (L)
20	+ (I)	70	- (L)	120	- (L)
21	- (L)	71	- (L)	121	- (L)
22	- (L)	72	- (L)	122	(*)
23	+ (I)	73	- (L)	123	(*)
24	- (L)	74	(*)	124	(*)
25	- (L)	75	(*)	125	(*)
26	- (L)	76	+ (I)	126	- (L)
27	- (L)	77	- (L)	127	- (L)
28	- (L)	78	- (L)	128	- (L)
29	- (L)	79	- (L)	129	- (L)
30	+ 0)	80	- (L)	130	- (L)
31	- (L)	81	- (L)	131	- (L)
32	- (L)	82	- (L)	132	- (L)
33	+ (I)	83	- (L)	133	- (L)
34	- (L)	84	- (L)	134	- (L)
35	- (L)	85	- (L)	135	- (L)
36	- (L)	86	- (L)	136	- (L)
37	- (L)	87	- (L)	137	- (L)
38	- (L)	88	- (L)	138	- (L)
39	- (L)	89	- (L)	139	- (L)
40	- (L)	90	- (L)	140	- (L)
41	- (L)	91	- (L)	141	- (L)
42	+ 0)	92	- (L)	142	- (L)
43	- (L)	93	+ (I)	143	- (L)
44	- (L)	94	- (L)	144	- (L)
45	- (L)	95	- (L)	145	- (L)
46	- (L)	96	- (L)	146	- (L)
47	- (L)	97	- (L)	147	- (L)
48	- (L)	98	- (L)	148	- (L)
49	- (L)	99	- (L)	149	- (L)
50	- (L)	100	- (L)	150	- (L)

(L)= Lipase positive, - (L) = Lipase negative, + (I)=*Bacillus* insert present and (*)= Contaminated.

Table 4.4:Observations recorded from screening of XLRH genonlic library.

Colony No.	Reaction	Colony No.	Reaction	Colony No.	Reaction
1	- (L)	51	- (L)	101	- (L)
2	- (L)	52	- (L)	102	- (L)
3	- (L)	53	+ (I)	103	- (L)
4	- (L)	54	- (L)	104	- (L)
5	- (L)	55	+ (I)	105	- (L)
6	+ (I)	56	- (L)	106	- (L)
7	- (L)	57	- (L)	107	(*)
8	+ (I)	58	- (L)	108	- (L)
9	+ (I)	59	- (L)	109	- (L)
10	- (L)	60	- (L)	110	- (L)
11	- (L)	61	- (L)	111	- (L)
12	- (L)	62	- (L)	112	- (L)
13	- (L)	63	- (L)	113	- (L)
14	- (L)	64	- (L)	114	- (L)
15	+ (I)	65	- (L)	115	- (L)
16	+ (L)	66	- (L)	116	- (L)
17	- (L)	67	- (L)	117	- (L)
18	- (L)	68	- (L)	118	- (L)
19	- (L)	69	- (L)	119	- (L)
20	+ (I)	70	- (L)	120	- (L)
21	- (L)	71	- (L)	121	- (L)
22	- (L)	72	- (L)	122	- (L)
23	+ (I)	73	- (L)	123	- (L)
24	- (L)	74	- (L)	124	- (L)
25	- (L)	75	- (L)	125	- (L)
26	- (L)	76	- (L)	126	- (L)
27	- (L)	77	- (L)	127	(*)
28	- (L)	78	- (L)	128	(*)
29	- (L)	79	- (L)	129	- (L)
30	+ (I)	80	- (L)	130	- (L)
31	- (L)	81	- (L)	131	- (L)
32	- (L)	82	- (L)	132	- (L)
33	- (L)	83	- (L)	133	- (L)
34	- (L)	84	- (L)	134	- (L)
35	- (L)	85	+ (I)	135	- (L)
36	- (L)	86	- (L)	136	- (L)
37	- (L)	87	- (L)	137	- (L)
38	- (L)	88	- (L)	138	- (L)
39	- (L)	89	+ 0)	139	- (L)
40	- (L)	90	- (L)	140	- (L)
41	- (L)	91	- (L)	141	- (L)
42	- (L)	92	- (L)	142	- (L)
43	+ (I)	93	- (L)	143	- (L)
44	- (L)	94	- (L)	144	- (L)
45	- (L)	95	- (L)	145	- (L)
46	- (L)	96	- (L)	146	- (L)
47	- (L)	97	- (L)	147	- (L)
48	- (L)	98	- (L)	148	- (L)
49	(*)	99	- (L)	149	- (L)
50	(*)	100	- (L)	150	- (L)

+ (L)= Lipase positive, - (L) = Lipase negative, + (I)= *Bacillus* insert present and (*) = Contaminated.

lipase negative, to look for the inserts of *Bacillus* strains that were used during the investigation. Plasmid DNA when electrophorased on agarose gel (0.7%) revealed variation in the molecular weights when compared with the standard mol. wt. ladder thus confirming the differences in the insert size [Fig.4.4 (A) and (B), Fig. 4.5 (A) and (B)].

The gel pictures [Fig.4.4 (A) and (B), Fig. 4.5 (A) and (B)].represents the electrophoretic variation in the isolated plasmid DNA from the genomic library generated with the *Bacillus subtilis* (BB-I) and *Bacillus licheniformis* (XLRH) genomic DNA. The differences in the electrophoretic mobility of the plasmid DNA containing the insert which represent lipase positive recombinant clones BB-14 *(Bacillus subtilis* BB-I) and XL-16 *(Bacillus licheniformis* XLRH) are comparable to those representing the transformants which were recombinants but were lipase negative. The variation in their respective molecular weight confirmed the presence of foreign DNA inserts in the plasmids of all the transformants.

To further confirm the presence of foreign DNA inserts, the plasmid DNA of the transformants were restriction digested with Hind III. to release the foreign DNA insert from the vector used for cloning. Restriction digestion analysis was carried out on 0.7% agarose gel. λ-DNA ladder was used as a molecular weight marker and the digested pUC19/Hind III DNA was used as control. The DNA insert thus released showed a considerable variation in their molecular wt. and thus in their electrophoretic mobility, confirming the differences in the size of DNA fragments.

Restriction digestion analysis of the same set of plasmid DNA from BB-14 and XL-16 which showed lipase positive reaction, and also from other recombinant clones are presented in [Fig 4.6 (A) and (B), Fig. 4.7 (A), (B) and (C)]. One of the transformants (BB-14) of*Bacillus subtilis* (BB-I) and that from *Bacillus licheniformis* (XLRH) i.e. XL-

Recombinant Plasmids Isolated from BB-1 Genomic Library

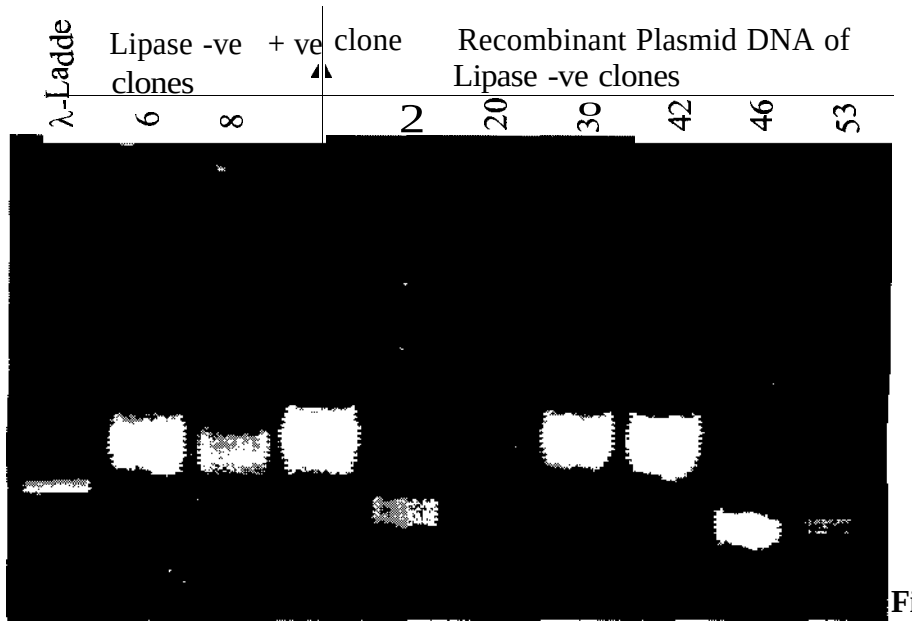


Fig. 4.4 A

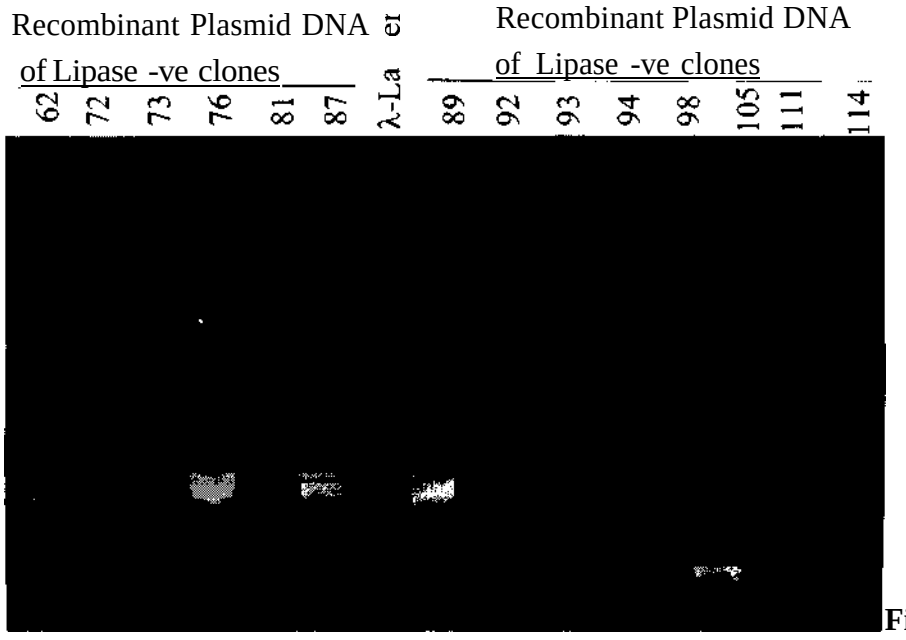


Fig. 4.4 B

Recombinant Clones Isolated from XLRH Genomic Library

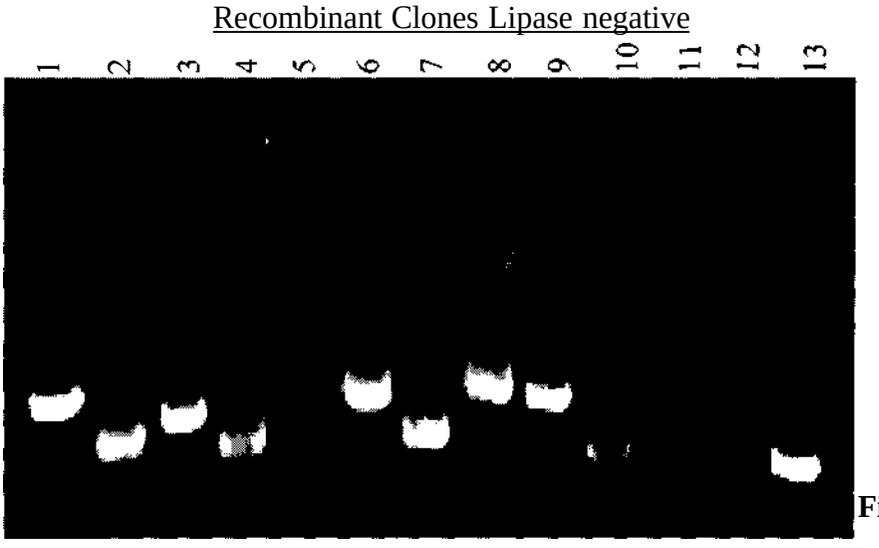


Fig. 4.5 A

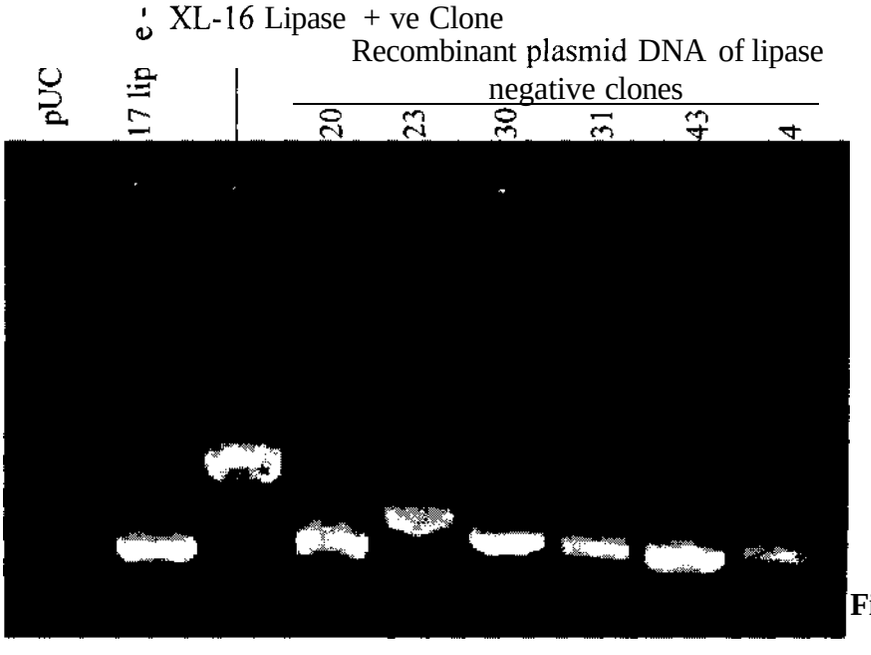
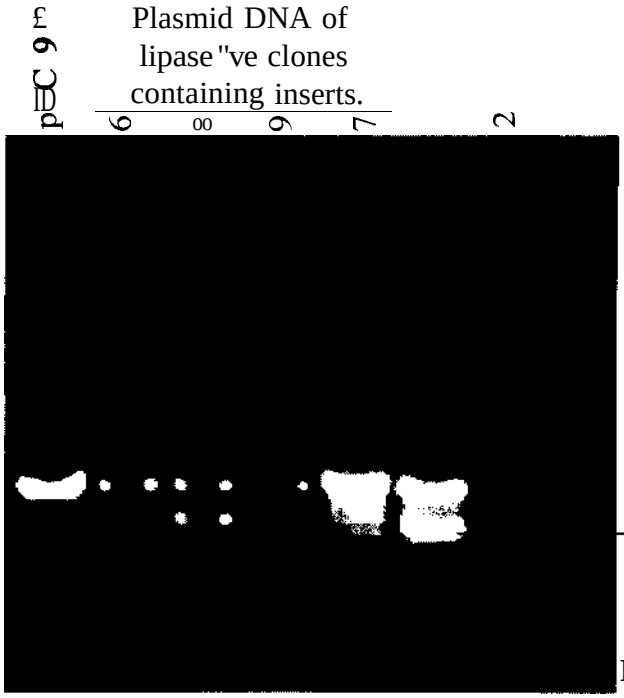


Fig. 4.5 B

Restriction Digestion Analysis of XLRH transformants.



2Kb insert size of XL-16 Lipase + ve clone used for sequencing.

Fig. 4.6 A

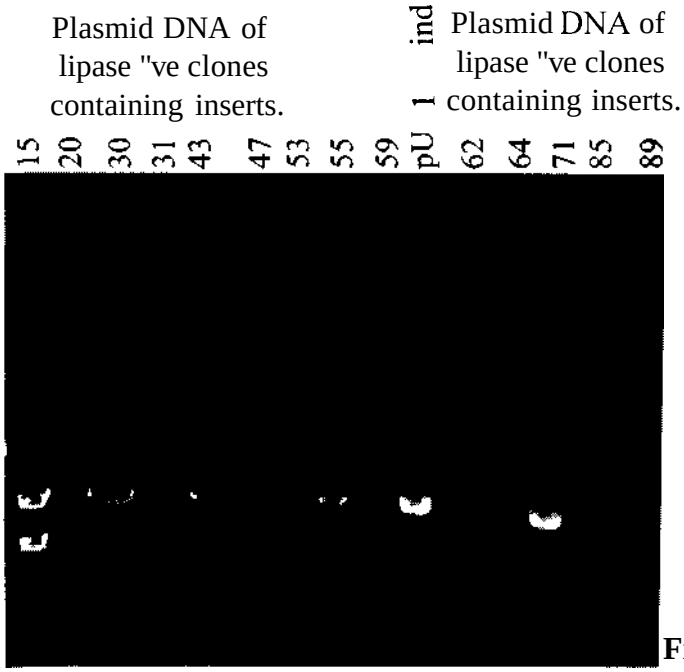


Fig. 4.6 B

Restriction Digestion Analysis of BB-1 Transformants

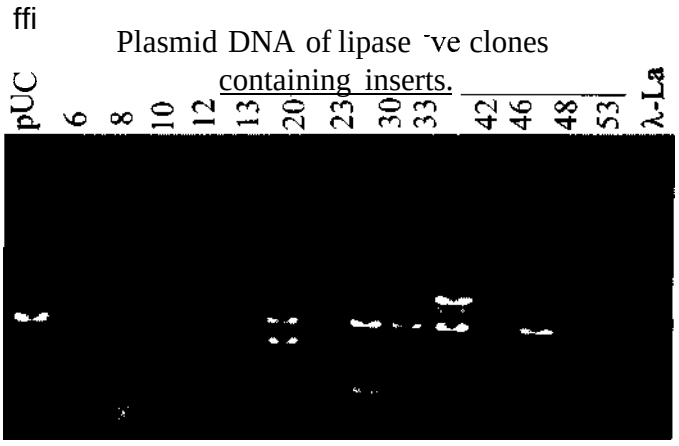
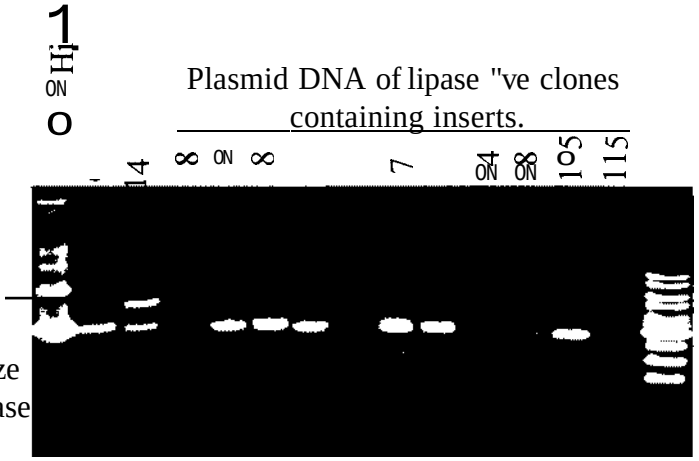


Fig. 4.7 A



5Kb insert size of BB-14 lipase + ve clone.

Fig. 4.7 B

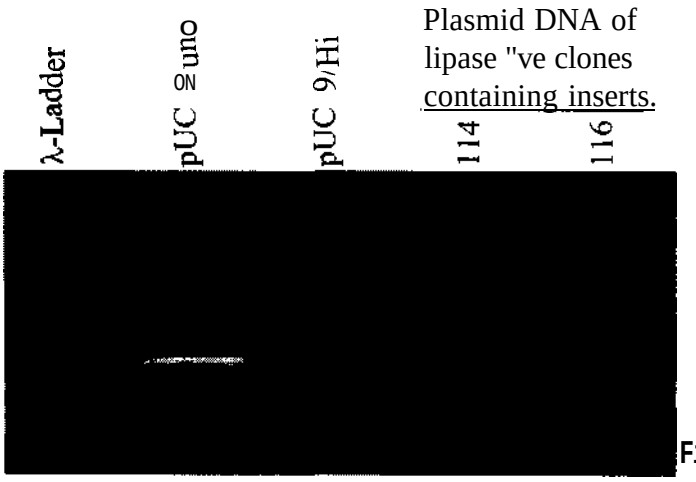


Fig. 4.7C

16 that reacted positive for lipolytic activity showed the presence of a 5Kb and 2Kb DNA insert size and were later used for sequencing.

4.6 Automation sequencing and sequence analysis

Plasmid DNA from the transformants, (BB-14 and XL-16 which showed lipase positive reaction), and of an untransformed blue colony (XL-31) were selected for sequencing.

The sequences obtained after automation sequencing for BB-14, XL-16 and XL-31 are detailed as below. These sequences were fed into the Blastn program to analyze for the possible sequence homologies with the known bases sequence of lipase genes from other microorganisms available in the data-banks of EMBL and GenBank on the internet at the web site detailed elsewhere in materials and methods (section 3.10.1).

1. Sequence analysis of 5 Kb fragment of *Bacillus subtilis* (BB-1) in *the* BB-14 transformant present in pUC 19 plasmid cloning vector

CTCCCGGGATTTCTGAGTCCCCTGAGGCATGAAGCTTGTATTACAGCCTTGGCGACAAAGAGA
GCCGAACCGCTCACAGAGAAGCGCTGATCCGCCACTTTGAGCCGCGGATCGACGAGTTTTGTT
CA**GACTGTCAAAAACGGC**TCCGGCAAAACCCGCTGCGGATTCTGGACTGCAAAAAAGACAG
GGATC**ATGAACTGCTGAAAA**CGGCTCCGTCGATTCTTGAC

TACTTAAATGAAGAATCTAAAGCATATTTTGCAAAGGTACAGCAATATCTGACGGACATCGG
CATTGCATTTGAGG **Putative base sequence of lipase**
4

TCGATCCGAATCTGGTGAGAGGGCTTGACTATTACAATCACACGGCCTTTGAAATCATG
AGCAATGCCGAAGGCTTCGGAGCCGATTACGACGCTTGCCGGCGGGGGGCCGCTATGA
CGGCCTTACGGAGGAGTTCGGCGGTCCGAAAGCGCCGGGCATCTGGCTTTGCGATGAG
CATCGAACGC

CTTTCTCGCCGCACTTGGATGCTTGAAAATGTAAAAAGTCCGGAGCCGGATGAAAGGGCATTT
GACTTGCTATATCCGTGACAACTTTGG**CCGACAAAGCCCAAA**AGACTATTCCAGTATCC
CCTTT

A 615 base sequence that was obtained by the automated sequencing of the recombinant plasmid from BB-14 was queried for similarity with *Bacillus* sp and then blastn search, was made. The results obtained are detailed in Table 4.5.

Table 4.5: Results of the sequence similarity search.

S. No	Query Sequence (bp)	*Base sequence	Result (Homology with)	Score (bits)	e-value
1	130-144	1	*Bacillus subtilis* complete genome (Section 2 of 415810)	30	4.8
2	193-207	2	*Bacillus subtilis* complete genome (Section 6 Of 1209940)	30	4.8
3	228-242	3	*Bacillus subtilis* complete genome (Section 11 of 2207900)	30	4.8
4	304-484	4	*Bacillus subtilis* complete genome (Section 15 of 3013540)	98	2e-20
5	579-593	5	*Bacillus subtilis* complete genome (Section 16 of 3213410)	30	4.8

**** Box in bold facesindicated in sequence and numbered Ito 5***

To identify the putative lipase base sequence within the 615 base sequence, the sequenced data was fed and compared with bacteria (ORGN). A homology of 34 bits with *Neisseria meningitidis Lip A* and *Lip B* genes encoding *Lip A* and *Lip B* proteins was revealed and thus was designated as LIP 2 (339-355 of the query sequence highlighted).

BB-14 (Lip2)	Query Sequence(615 bp)
Score	34.2 bits (17)
Identities	17/17 (100%)
Query	339 AA TC AC A C G G C C T T T G A 355
Subject	4253 AA T C A C A C G G C C TT TG A 4237

2. Sequence analysis of 2 Kb fragment of *Bacillus licheniformis*(XLRH) in the XL-16 transformant present in pUC 19 plasmid cloning vector

TCAGGTCCCGGGTCCGGCACGATTACTGGCCGTCGTTTACAACTTCGTGACTGGGAAAACCCT

GGCGTTCCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGTTGGCTGTAATAGCGAA

GAGGCCCGCACCGATCGCCCTTCCCAACAGATGCGCAGCA**CCTGAGAGATCCTGTCAT**

AATTTCCCCAAAGCGTAACCATGTGTGAATAAATTTTGAGCTAATAGGGT

TGCAGCCACGAGTATGTCTTCCCTTGCTATTGCGTAGCCAGAATGCCGC

AAAACTTCCATGCCTAACCGAACTGTTGAGAGTACGTTTCCATTTCTCGA

CTGTGTTAGCCTGGAAGTGCTTGTCCCAACCTTGTTTTCTGAGCATTGAA

CCGCCCGTAAGCCCAACATGTTAGTTGAAGCATACATGGCGATTAGCTG

CATGATTATACAAAAC

Plasmid DNA from XL-16 a transformant of *Bacillus licheniformis* (XLRH) genomic library was sequenced. Automation sequencing revealed a base sequence of 448. After blastn search, the query sequence showed homology of 357 bits with *Bacillus megaterium* α-amylase gene. The expectancy (e-value) found was highly significant (4e-97) (Table 4.6).

XL-16 (Lip1) = 448 bases

Table 4.6: XL-16 sequences showing significant alignments.

S.No	Query Sequence	Base Sequence	Result (Homology with)	Score (bits)	**e-value**
1	167-438	Indicated in bold face in the sequence data of XL- 16	*Bacillus megaterium* alpha-amylase gene	357	4e-97

3. Sequence analysis of XL-31 untransformed pUC19 Vector

ACTTTTCGGAACTTGCATGCAGGTCTCTGCAGTACCCGGGCCCGGGATCCGATATCTA

GATGCATTCTTTGAGGTACCGATTTAACC**TTCACTGGCCGTCGTTTTACAACGTAC**

GCAGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCAC

ATCCCCCTTTCGCCAGCTGGCCGTAATAGCGAAGAGGCCCGCACCGATC

GCCCTTCCCAACAGTTGCTTTTTTTTGAATGGCGAATGGCTGCCTGATGC

GGAATTTTCTCCTTACGCATCTGTGCGGTCTTTCACACCTGCATATGGTG

CACTGCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCACCC

V

GACACCCGCTAACACCCGCTGACGGCGCTCTGACAGGGGCTTGCATTGCTCCCCGG
CCATCCTGCTTACCAGGACAAAGCATGGGAACCCGTTTCC

A sample of untransformed blue colony representing pUC 19 vector DNA was also selected for sequencing to confirm the results about the authenticity of the vector used. Sequencing of the sample revealed a base sequence of 457 bases and showed 391 bits homology with pUC19 cloning vector Table 4.7.

XL 31=457 bases

Table 4.7: XL-31 Sequences showing significant alignments.

S.No	Query Sequence	Base Sequence	Result (Homology with)	Score (bits)	e-value
1	88-385	Indicated in bold face in the sequence data of XL 31	Cloning vector pUC 19 sequence	391	e-106

4.7 DNA cassettes for enhanced enzyme activity

The main objective of designing the DNA cassettes was to enhance the expression of the gene encoding the lipase protein. The 5 Kb insert size of BB-14 that showed a 34 bits homology with lipase was used for ligating it in the designed expression vector for studying its enhanced expression in the *E. coli* (sure) host.

The 5 Kb insert size was ligated to the expression vector (pFLAG-ATS) having a strong *tac* promoter for the lipase gene. The recombinant expression vector was transformed into *E.coli* competent cells. Large number of ampicillin resistant transformants were obtained which were then replica-plated on LB/Tributyrin/Agar plates to observe the clearing zone formation. Poor intensity and the reduced size of clearing zone was observed as compared to that of parental culture and BB-14 transformant from the genomic library *of Bacillus subtilis* (BB-I).

CHAPTER-V

DISCUSSION

Bacteria produce and secrete lipases, which can catalyze both the hydrolysis and the synthesis of long chain acylglycerols. Lipases are the enzymes which in contrast to esterases, become active drastically when absorbed on to an oil water interface and do not hydrolyse soluble substrates. The increase in enzymatic activity is triggered by structural rearrangement of the lipase active-site region (Brzozowski *el al.*, 1991; Derewenda *el al.*, 1992). These reactions usually proceed with high regioselectivity and enantioselectivity, and therefore, lipases have become very important stereoselective biocatalysts used in organic chemistry. These are ubiquitous enzymes of considerable physiological significance as well as industrial potential. Enzymes with industrial potential are usually obtained from microorganisms which produce a wide spectrum of extracellular lipases. Many are active in organic solvents where they catalyze a number of useful reactions including trans-esterification, regioselective acylation of glycols and mentols, even synthesis of peptides (Valivety *el al.*, 1992). The use of lipases especially in household detergents has been, however, restricted at the moment because of their instability under alkaline conditions. Alkaline lipases active at pH above 7.0 are being reported from various bacterial species including *Bacillus* (Sidhu *el al.*, 1998; Wang *et al.*, 1995), *Staphylococcus warneri* (Telefoncu *et al.*, 1990) and *Pseudomonas pseudoalcaligenes* (Lin *et al.*, 1996). The world of bacterial lipases is rapidly expanding. An impressive number of lipase genes have been identified and many lipase proteins were biochemically characterized. Undoubtedly, there is a steadily increasing demand to identify,

characterize and produce lipases for a variety of biotechnological applications, with special emphasis on enantioselective biotransformants. Therefore, as a first step, a standard assay system should be developed, that allows testing hydrolysis and synthesis reaction catalysed by a given lipase. At least one system that allows for heterologous expression and secretion of different lipases, and a data bank should be built comprising of sequences of available genes and proteins.

The model organisms *Bacillus subtilis* is gram positive, in which a number of genetic tools have been developed. In addition, the genetics of other extracellular hydrolytic enzymes e.g. protease and amylase has been investigated thoroughly (Henner *el al.*, 1987 and 1988, Weickert *et al.*, 1990). The genus is also well known for their lipase activities and thus are the potential source for its endogenous lipase genes. Thus, the present investigation was carried out with the identification of lipase positive strains from *Bacillus subtilis* and *Bacillus licheniformis*. The identification of positive strains from these isolates prompted us for molecular cloning, expression in *E. coli* and nucleotide sequence of the putative lipase gene.

5.1 Identification *of Bacillus* strains with lipolytic activity

Colonies of organism causing clearing of the plate medium are regarded as putative lipase producers. The lipolytic activity of the organisms is also detected on the basis of clearing of the agar plates containing tributyrin (Arima *et al.*, 1972), or Tweens in the nutrient medium in which the detection of opaque zone around the colony is the proof of lipase activity (Sierra, 1957). The views arc in support of the present investigation that the strains identified each from *Bacillus subtilis* and *Bacillus licheniformis* coded as BB-I and XLRH respectively and

only two transformants BB-14 containing a chromosomal DNA fragment of *Bacillus subtilis* (BB-I) and XL-16 carrying chromosomal DNA fragment from *Bacillus licheniformis* (XLRH) showed a distinct clearing zone as a result of lipolytic activity on LB/Tributyrin/Tween agar plates, following titremetry and agar diffusion methods and are thus a suitable source of lipase producing microorganisms.

5.2 Molecular cloning, sequence homology and expression of lipases

5.2.1 Molecular cloning

5.2.1.1 Plasmid cloning vectors

Since the early days of molecular cloning, bacterial plasmids have received much more attention because of their usefulness as vectors in recombinant DNA experiments. From the identified sources of lipases in the present investigation a high molecular weight chromosomal DNA isolated from *Bacillus* strains (BB-I and XLRH) was purified, and 1-6 Kb fragments thus generated through restriction digestion was ligated to the linearized eluted fragment of pUC19 (2.9Kb) plasmid cloning vector. Plasmid cloning vector pUC 19 has been successfully used by Jorgensen *et al.* (1990), clone *Lip A* gene encoding an extracellular lipase from *Pseudomonas cepacia* and a lipase gene from *Bacillus stearothermophilus* by Kim *el al.,* (1998). Another lipase gene *(lip)* and its activator gene *(act)* on a 2.9Kb BglII-EcoRI fragment from *Pseudomonas* sp. KW1-56 were cloned in *E.coli* using pUC19 has been reported by Iizumi *el al.* (1991). To prepare a partial genomic library of the genomic DNA from *Pseudomonas putida* into JM-109 cells of £ *coli*, pUC 19 cloning vector was used Ozaki *et al.* (1994). The choice of pUC 19 stems primarily from its unique

properties. It contains portions of pBR 322 and M13mp19 and can be amplified with chloramphenicol. It is a small, high copy number *E.coli* plasmid cloning vector which carries a 54 bp multiple cloning site polylinker. It has (1) *bla* gene, coding for β-lactamase that confers resistance to ampicillin (source-plasmid pBR 322). It differs from that of pBR 322 by two point mutations; (2) Region *of E.coli* operon *lac* containing CAP protein binding site, promoter P *lac* lac repressor binding site and 5′ terminal part of the *lac Zα* gene encoding the N-terminal fragment part of β-galactosidase (source-M13mp18/19).

Besides pUC 19 there are several other **Cloning vectors used for cloning microbial lipases (detailed elsewhere in review section 2.8.1.1 Table** 2.3). The design and construction of cloning vectors has now become complex and highly sophisticated area of study, yielding a vast amount of information and greater number of specialised vectors for investigations to use.

5.2.1.2 **Electrotransformation**

The process of transformation was the first genetic process to be observed in bacteria and yet it remains one of the most remarkable transfer mechanisms. Electroporation is rapidly becoming one of the most efficient and versatile techniques for introduction of DNA into the cells. This technique involves the application of brief, high intensity electric fields to cells to reversibly permeabilize the membrane to exogenous molecules (Knight, 1981).

Competent cells of *E.coli* (JM-109) were transformed with the plasmid bearing the ligated chromosomal DNA (1-6Kb) fragment. The fragments were derived from *Bacillus subtilis* (BB-I) and *Bacillus licheniformis* (XLRH) separately following electrotransformation technique thus resulting in a higher

frequency of stable transformants at a frequency of 1 x 10 colonies / ug of DNA and 2 x 10^5 colonies / μg of DNA respectively. The time constant and thus the number of transformed cells varied with the source of genomic DNA fragments used. Observations in the present investigation supports the views made by Dower *el al.* (1988), that the concentration of DNA greatly affects the recovery of transformants. With *E.coli*, the frequency of transformation (transformants/survivor) was found to be strictly dependent on DNA concentration. At higher concentration upto 80% of the survivors were transformed. The difference in the quality and the concentration of the genomic DNA obtained and used for preparing genomic library from the *Bacillus subtilis* and *B. licheniformis* may have resulted in the differences in the frequency of stable transformants

Electroporation is rapidly becoming the method of choice for transforming gram-positive microorganisms which previously were difficult to transform via protoplast/PEG methods (Chassy and Flickinger 1987; Fielder and Writh 1988; Miller *et al.*, 1988; Powell *et al.*, 1988; Belliveau and Trevors 1989; Brigdi *et al.*, 1990; Wei *et al.*, 1995; Kelly *et al.*, 1996; Ohse *et al.*, 1997).

5.2.1.3 Screening of the genomic library

Following the demonstration that a gene originating from *Staphylococcus aureus* can function in an unrelated bacterium, *Escherichi coli*, it was widely assumed that genes from any bacterium could be expressed in any other. This idea was strengthened by the observation that genes from two lower eukaryotes, *Saccharomyces cerevisiae* (Struhl *et al.*, 1976) and *Neurospora crassa* (Vapnek *et al.*, 1977) are also expressed in *E. coli.* Today *E. coli* is a system that allows for

heterologous expression of the gene of interest. A total of three hundred transformants, representing one hundred fifty white colonies each of *Bacillus subtilis* (BB-I) and *Bacillus licheniformis* (XLRH) were selected to develop a partial genomic library of the two likely lipase positive strains *Bacillus subtilis* (BB-I) and *Bacillus licheniformis* (XLRH) in *E. coli* (JM-109). The transformants were then screened on LB/Tributyrin/Agar plates and two transformants BB-14 containing a chromosomal DNA fragment *of Bacillus sublilis* (BB-1) and XL-16 carrying chromosomal DNA fragment from *Bacillus licheniformis* (XLRH) were identified as the likely source of cloned gene fragment encoding putative lipase gene. Genomic constructs in *E. coli* from several microorganisms producing lipases have been reported (Aoyama *et al.,* 1988; Chung *et al.,* 1991; Alberghina *et al.,* 1991; Tan and Miller, 1992) and lipolytic activity of the organisms is also detected on the basis of clearing of the agar plates containing tributyrin (Arima *et al.*, 1972)

The genomic DNA of lipase producing *Rhizopus delemar* ATCC 34612 was isolated, digested with Mbol, ligated with plasmid pBR 322 (4.3Kb) and transformed into *E.coli* C600 and was verified by colony hybridization using biotinylated fungal DNA as a probe (Haas *et al.,* 1990).

5.2.1.4 Automation sequencing and molecular sequence homology of lipases

Determination and comparison of specific activities and substrate specificities of different lipases are absolutely required to investigate the physiological function of lipases as well as to judge their usefulness for biotechnological applications. Fifty three sequences of bacterial lipases and esterases are compared and classified according to conserved sequence motifs and

the biological properties (Arpigny and Jaeger 1999) which permits the identification of newly isolated lipolytic enzymes.

A 615 base sequence that was obtained by the automated sequencing of the recombinant plasmid from BB-14 was queried for similarity with *Bacillus* sp this confirmed the fragment which was cloned from *Bacillus subtilis* (Homologous sequences are detailed elsewhere in result section 4.6). Frosch and Muller (1993) had sequenced lipase gene from *Neisseria meningitidis* by developing a cosmid library and reported the presence of *LipA* and *LipB* genes encoding their respective protein and were responsible for the phospholipid modification of capsular polysaccharide. The putative gene fragment which was sequenced in the present investigation revealed a homology of 34 bits with *Lip A* and *Lip B* genes and was designated as *LIP 2.*

BB-14 Query (615 bases) **Fragment of *B. subtilis* (BB14 transformant)**
339 AA TC AC A C G G C C T T T G A 355
4253 A A T C A C A C G G C C T T T G A 4237
Subject *(Neisseria meningitidis)*
Identities 17/17 (100%)

Plasmid DNA from XL-16 a transformant of *Bacillus licheniformis* (XLRH) revealed a base sequence of 448 bases. The query sequence showed homology of 357 bits with *Bacillus megaterium* α-amylase gene with a highly significant expectancy (e-value) of 4e-97.

There are a large number of reports published, on the molecular cloning and sequencing of the lipase gene from different microbial strains. The identity of these genes were confirmed by comparing them with the homologous lipases sequences (Nucleotide or aminoacid) from other organisms, (Lee and Landolo

1985; Gilbert, 1993; Kim *el al.*, 1996; Gotz *et al.*, 1985 & 1998; Nikoleit *et al.*, 1995).

Thus the fragments sequenced from the transformants BB-14 and XL-16 confirms the presence of putative lipase and alpha amylase gene respectively based on their respective homologies with *Neisseria meningitidis* and *Bacillus megaterium* gene sequences. The putative sequence that was identified can be used to design primers and probes. The identification of these cloned fragments can thus permit the identification of newly isolated lipolytic enzymes and can also be useful in determining and comparing specific activities and substrate specificities among different lipases. Thus, the data bank which thus will be generated is absolutely required to investigate the physiological function of lipases as well as to judge their usefulness for biotechnological applications.

5.3 **DNA cassettes for enhanced enzyme activity**

Lipase enzymes found in many different organisms are frequently used during the synthesis of pharmaceutical compounds for racemate resolution to yield a single, desired stereospecific isomer. The use of lipase for large-scale process is expensive and aims to develop an in-house source of lipase. The cloned bacterial lipase genes in many cases could directly express in the host organism. However, some but not all Upases require a secondary gene for their activation and has been reported by several workers (Chung *et al.*, 1991; Cino, 1999; Goetz *el al,* 1988; Jorgensen *el al.*,1991; Hobson *et al.*, 1993; Iizumi *et al.*, 1991; Hirayama 1993; Yoshikawa *et al.*, 1992 & Wohlfarth *et al.*, 1992; Ihara *et al.*, 1995; Frenken *et al.*, 1993).

The putative Hpase gene cloned and sequenced from BB-14 (5-Kb) was ligated to the expression vector (pFLAG-ATS) which has a strong *lac* promoter for the Hpase gene. The recombinant expression vector was transformed into *E.coli* (Sure) competent cells. Poor expression of the cloned putative Hpase gene was observed as compared to that of parental culture and BB-14 transformant from the genomic library of *Bacillus subtilis* (BB-I).

Jorgensen *et al.* (1991), indicated that a secondary gene product is required for the correct folding as well as for the hyperexpression of the Hpase gene in heterologous hosts. Rua *et al* (1998), reported an efficient expression system for the previously only weakly expressed thermophilic Hpase BTL2 gene *(Bacillus thermocatenulatus* lipase 2) was developed for the production of large amounts of lipase in *Escherchia coli* BL321, DH5-alpha and JM 105. The gene was isolated from plasmid pLIP 2 and ligated into the plasmid pCYTEXPl (pT1) expression vector downstream of the temperature-inducible lambda promoter PL.

Poor expression of the cloned putative sequence in the present investigation might be due to the following reasons 1) A secondary gene might be required for the enhancement of the lipase gene expression, as reported by several workers might be required. 2) A secondary gene product might be required for the correct folding as well as for the hyperexpression of the Hpase gene in heterologous hosts. 3) The gene might have been inserted in an inappropriate place which might have affected the expression. 4) The fragment cloned being putative might be responsible for poor expression.

SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

CHAPTER VI

SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

Organisms producing lipases are widespread and have been found in various natural habitats. In eukaryotes, lipases are involved in various stages of lipid metabolism including fat digestion, adsorption, reconstitution and lipoprotein metabolism. In plants, Upases are found in energy reserve tissues. However, enzymes with industrial potential are usually obtained from microorganisms that produce a wide spectrum of extracellular Upases.

Enzyme-mediated reactions are attractive alternative to tedious and expensive chemical methods. The demand of industrial enzymes in general, and Upases in particular, is ever increasing owing to their application in a wide variety of processes. In recent years, genetic engineering techniques have been applied in the mass production of these enzymes. *Bacillus* strains are well known for their lipase activities and thus are the potential source for its endogenous lipase genes which prompted us to carry out the present investigation with the identification of lipase positive strains from *Bacillus sublilis* and *Bacillus licheniformis*,formation of genomic library in a suitable host, its screening for lipase activity, sequencing and sub-cloning in a designed expression vector for its enhanced expression in *E. coli*.

6.1 Summary

6.1.1 Identification of *Bacillus* strains with lipolytic activity *Bacillus* strains were screened and identified as lipase positive from *Bacillus subtilis* (BB-I) and *Bacillus licheniformis* (XLRH), were selected for isolation and sequencing of the putative lipase genes.

6.1.2 Development of partial genomic library and selection of recombinant clones Partial genomic library was constructed in *E. coli* (JM-109) from *Bacillus subtilis* (BB-I) and *Bacillus licheniformis* (XLRH). The time constant achieved

for DNA uptake and the number of transformants obtained varied with genomic DNA of the strain used. The number of transformants using the *Bacillus subtilis* (BB-I), genomic DNA fragments (1-6 Kb), were less (1×10^5 colonies / μg of DNA), and was just doubled when the the genomic DNA (1-6 Kb) fragments of *Bacillus licheniformis* (XLRH), (2×10^5 colonies / μg of DNA) was used.

6.1.3 Screening of the recombinants for lipolytic activity Three hundred transformants, representing one hundred fifty white colonies each of *Bacillus subtilis* (BB-1) and *Bacillus licheniformis* (XLRH) were screened on LB/tributyrin/tween agar plates. Only two transformants BB-14 containing a chromosomal DNA fragment of *Bacillus subtilis* (BB-I) and XL-16 carrying chromosomal DNA fragment from *Bacillus licheniformis* (XLRH) were identified as lipase positive on LB/Tributyrin/Tween agar plates.

6.1.4 Analysis of recombinant plasmid DNA isolated and its restriction digestion Electrophoretic variation in the isolated plasmid DNA (digested and undigested with restriction enzymes) of the transformants from the genomic library generated with the *Bacillus subtilis* (BB-1) and *Bacillus licheniformis* (XLRH) genomic DNA was observed. The transformants (BB-14) of *Bacillus subtilis* (BB-I) and that from *Bacillus licheniformis* (XLRH) i.e. XL-16 that reacted positive for lipolytic activity showed the presence of a 5Kb and 2Kb DNA insert size and were later used for sequencing.

6.1.5 Automation sequencing and analysis of sequences Plasmid DNA from the transformants, (BB-14 and XL-16 which showed lipase positive reaction), and of an untransformed blue colony (XL-31) were sequenced which are as below.

a BB-14 present in the plasmid pUC 19 cloning vector The putative lipase base sequence (AATCACACGGCCTTTGA) within the 615 base sequence of BB-14 was identified by comparison in the bacteria (ORGN). A homology of 34 bits with *Neisseria meningitidis* Lip A and Lip B genes encoding Lip A and Lip B proteins was revealed and thus was designated as LIP 2.

Q 2) XL-16 in the plasmid pUC 19 cloning vector. Automation sequencing of plasmid DNA from XL-16 a transformant revealed a base sequence of 448bp. The query sequence showed homology of 357 bits with *Bacillus megaterium* α-amylase gene with expectancy (e-value) of 4e-97.

a 3) Sequence analysis of XL-31 untransformed pUC19 Vector. Sequencing of the plasmid from the untransformed blue colony revealed a base sequence of 457 bases.and showed 391 bits homology with pUC19 cloning vector thus confirming the authenticity of the vector used.

6.1.6 DNA cassettes for enhanced enzyme activity Poor expression of the putative lipase sequence was observed when it was inserted into the pFLAG-ATS expression vector even in the presence of strong *lac* promoter.

6.2 Conclusions

Determination and comparison of specific activities and substrate specificities of different lipases are absolutely required to investigate the physiological function of lipases as well as to judge their usefulness for biotechnological applications. Putative gene fragments which were sequenced in the present investigation were designed as *LIP 2* and α-amylase gene based on their respective homologies with *Neisseria meningitidis* and *Bacillus megaterium* gene sequences.

The putative sequence that was identified can be used to design primers and probes. The identification of these cloned fragments can thus permit the identification of newly isolated lipolytic enzymes and can also be useful in determining and comparing specific activities and substrate specificities among different lipases. Thus the data bank which thus will be generated is absolutely required to investigate the physiological function of lipases as well as to judge their usefulness for biotechnological applications. During the course of present investigation we come up with were some of the unanswered problems which we propose herein and thus needs to be further investigated.

Why the strain showing lipase positive reaction on the media substrate which when later sequenced revealed to show a homology with the a-amylase?

Does the poor expression of the putative gene is determined by several of the following reasons?

1) A secondary gene might be required for the enhancement of the lipase gene expression
2) A secondary gene product might be required for the correct folding as well as for the hyperexpression of the lipase gene in heterologous hosts.

3) The gene might have been inserted in an inappropriate place which might have affected the expression.
4) The fragment cloned being putative might be responsible for poor expression.

6.3 Suggestions for future work

The world of bacterial lipases is rapidly expanding. An impressive number of lipase genes have been identified and many lipase proteins were biochemically characterised. The existing three dimensional structure of lipases allow the identification of domains and amino acid residues involved in substrate binding, catalysis and enantiopreference, thereby enabling researchers to tailor lipases for selected applications by using site-directed mutagenesis. The creation of lipases with novel properties by directed evolution constitutes a more general approach.

- A system should be developed for the identification, and characterization of bio-transformants that produce lipases with enantioselectivity that can be used for a variety of biotechnological applications.
- A standard assay system should be developed, allowing one to test, hydrolysis and synthesis reaction catalysed by a given lipase.
- A system, that allows the heterologous expression and secretion of different lipases needs to be developed.
- A data bank should be built comprising sequences of available genes and specific activities and stereoselectivity, and options available to express and produce these lipases.

ABSTRACT

Name: **Sadiq Majeed**
Major: **Biotechnology**
Thesis Title: **Screening of the Genomic Gene bank for Identification and Cloning of Gene encoding enantio-specific Lipase Enzyme.**
Advisor: **Dr. A.** S. **Kotasthane**

Organisms producing lipases are widespread and have been found in various natural habitats. In eukaryotes, lipases are involved in various stages of lipid metabolism including fat digestion, adsorption, reconstitution and lipoprotein metabolism. In plants, lipases are found in energy reserve tissues. However. enzymes with industrial potential are usually obtained from microorganisms that produce a wide spectrum of extracellular lipases.

Enzyme -mediated reactions are attractive alternative to tedious and expensive chemical methods. The demand for industrial enzymes in general, and lipases in particular, is ever increasing owing to their application in a wide variety of processes. In recent years, genetic engineering techniques have been applied in the mass production of these enzymes. Bacillus strains are well known for their lipase activities and thus are the potential source for its endogenous lipase genes Thus the present investigation was carried out with the identification of lipase positive strain from *Bacillus subtilis* and *Bacillus licheniformis.*

Our present observation regarding the "***Screening of the Genomic Gene Bank for Identification and Cloning of Gene encoding enantio-specific Lipase Enzyme***" indicates that *Bacillus subtilis* (BB-I) and *Bacillus licheniformis*(XLRH), showed lipase activity and were selected for isolation and sequencing of the putative lipase genes. Partial genomic library was constructed in *E. coli* (JM-109) from *Bacillus subtilis* (BB-I) and *Bacillus licheniformis* (XLRH). Three hundred transformants, representing one hundred fifty white colonies each of *Bacillus subtilis* (BB-I) and *Bacillus licheniformis* (XLRH) were screened on LB/tributyrin/tween agar plates. Only two transformants BB-14 containing a chromosomal DNA fragment *of Bacillus subtilis* (BB-I) and XL-16 carrying chromosomal DNA fragment from *Bacillus licheniformis* (XLRH) were identified as lipase positive.

Automation Sequencing and Sequences analysis of plasmid DNA from the transformants, (BB-14 and XL-16 which showed lipase positive reaction), and of an untransformed blue colony (XL-31) was done. The putative lipase base sequence (AATCACACGGCCTTTGA) within the 615 base sequence of BB-14 was identified by comparison in the bacteria (ORGN). A homology of 34 bits with *Neisseria meningitidis* Lip A and Lip B genes encoding Lip A and Lip B proteins was revealed and thus was designated as LIP 2. Automation sequencing of plasmid DNA from XL-16 a transformant revealed a base sequence of 448bp. The query sequence showed homology of 357 bits with *Bacillus megaterium* a-amylase gene with expectancy (e-value) of 4e-97. Sequence analysis of XL-31 untransformed pUC19 Vector. Sequencing of the plasmid from the untransformed blue colony revealed a base sequence of 457 bases.and showed 391 bits homology with pUC19 cloning vector thus confirming the authenticity of the vector used.

Poor expression of the putative lipase sequence was observed when it was inserted into the pFLAG-ATS expression vector even in the presence of strong *tac* promoter.

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APPENDIX-I

Constituents of different buffers and solutions

A) Buffers

Name	Constituents
1M Tris.Hcl	Tris 121.1g/lt (pH 8.0)
TE buffer	Tris.Hcl (pH 8.0) 10mM EDTA (pH 8.0) 1mM
Tris Acetate EDTA (TAE buffer)	Tris base 242g/lt Glacial acetic acid 57.1 ml 0.5M EDTA (pH 8.0) 100ml
DNA loading buffer	Sucrose 40% (w/v) Bromophenol 0.25% Xylene cyanol 0.25%

B) Solutions

Name	Constituents	Use
0.5M Na EDTA	EDTA 186.1g/lt	Chellates metal ions.
RNase (10mg/ml)	10mg RNase A (Pancreatic) 1 ml of 10mM Tris.Hcl (pH 7.5) 1.5mM Nacl	Purification of DNA
Ethidium bromide (5mg/ml)	25mg Ethidium bromide 5ml sterile water	Staining of DNA
Lysozyme (20mg/ml)	100mg of lysozyme dissolved in TE (pH 8.0)	Cell wall lysis
IPTG (20mg/ml)	200mg of IPTG dissolved in 1ml of sterile water and filter sterilised.	Inducer for β-galactosidase enzyme.
X-gal (20mg/ml)	100mg of X-gal dissolved in 5ml of dimethylformamide.	Chromogenic substrate.
7.5 M ammonium acetate	578.1g/lt	Precipitation of DNA
Ampicillin (50mg/ml)	500mg of ampicillin dissolved in 100ml of sterile water and filter sterilised.	Antibiotic
GES reagent	5m Guanidine thiocyanate (60g) 0.1M EDTA (3.7g) sterile distilled water (20ml) 1.7ml of 30% sarcosyl	Denaturation of proteins
Solution-I	Glucose 1.8g 1M Tris.Hcl (pH 8.0) 5ml 0.5M EDTA (pH 8.0) 4ml	Maintains the stability of sphaeroplasts
Solution-II	0.2N NaOH 100ml 1% SDS 100ml Sterile water	Enhances cell lysis and denaturation of proteins
Solution-III	5M potassium acetate 60ml Glacial acetic acid 11.5ml Sterile water 28.5ml	Precipitation of DNA