PRODUCTION, PURIFICATION, CHARACTERIZATION OF BACTERIAL LIPASE AND ITS EXPLOITATION IN DETERGENT INDUSTRY

By

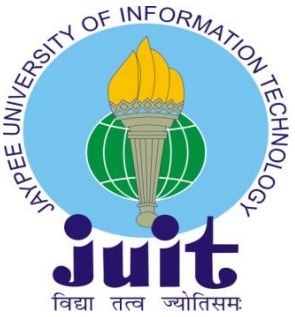
MAMTA CHAUHAN

A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY



JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY
WAKNAGHAT

OCTOBER, 2014

CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled "**Production, purification, characterization of bacterial lipase and its exploitation in detergent industry**", submitted by **Mamta Chauhan** at **Jaypee University of Information Technology, Waknaghat**, **India,** is a bonafide record of her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

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Date ( )

DECLARATION

I hereby declare that the work reported in the Ph.D. thesis entitled **"Production, purification, characterization of bacterial lipase and its exploitation in detergent industry"** submitted at **Jaypee University of Information Technology, Waknaghat India, is** an authentic record of my work carried out under the supervision of Dr. Vijay Kumar Garlapati. I have not submitted this work elsewhere for any other degree or diploma.

(Signature of the Scholar)

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ACKNOWLEDGEMENTS

The thesis dissertation marks the end of a long and eventful journey for which there are many people that I would like to acknowledge for their support along the way. As almost all PhD theses, this is no exception, as what the number of people that contributed to it is concerned and it is time to thank them for all they did for helping me to reach this goal.

To start with, my deepest thanks belong to my guide and supervisor Dr. Vijay Kumar Garlapati for his valuable guidance, help, encouragement and advice as my mentors, which made possible the successful completion of this work. I am privileged to have him as a source of knowledge for his valuable advice, constructive criticism and an authentic educator offering me a wise feedback.

I gratefully acknowledge the help rendered by Prof. R. S. Chauhan (HOD, Dept. of BT & BI) for his encouragement, timely help and cooperation throughout my research work.

I also wish my gratitude to Dr. Y. Medury (COO, Jaypee Education System) and Brig. (Retd.) Balbir Singh (Director, JUIT) for giving the opportunity to performing doctoral research work at Dept. BT & BI of JUIT. I would like to thank the authorities of Jaypee University of Information Technology, Waknaghat for providing the financial support during my research work.

I wish to convey my sincere thanks to all faculty members of Department of Biotechnology and Bioinformatics especially Prof. C. Tandon for helping in purification part, Dr. Gargi Dey, Dr. Pradeep Kumar Naik for their help and guidance at the various stages of this study. I also like to thank DPMC members Prof. T.S Lamba, Prof. C. Tandon, Dr. Karanjeet and Dr. Saurabh Bansal for their valuable suggestions. I Acknowledge Dr. Mani Shrivastava for bioinformatic work and Ms. Tamanna and Mr. Ashutosh for isolation and screening studies of lipase producing microbial strain

As always, it is impossible to mention everybody who had an impact to this work. However, there are those whose spiritual support is even more important. I have no words to extend my deep sense of gratitude to my loving parents, sisters who contributed their patience, moral support, encouragement, co-operation and throughout this thesis work. I can hardly able to

express my love to my sweet niece Vageesha and nephew Aryaveer whose innocence refreshed me during my difficult times. Above all I would like to acknowledge the tremendous sacrifices that my parents made to ensure that I had an excellent education. For this and much more, I am forever in their debt.

I thank all my friends Kirti, Shikha, Smriti, Rashmi, Swati, Dr. Mani and Manika and for the moral support and help provided by them during all ups and downs in this journey of research. 1 also extends my appreciation to Sharad, Varun, Amit and Anshu who willingly extended their help every time I required.

I am also thankful to all the members of technical and non-technical staff of the department, especially Mrs. Somlata Sharma, Mrs. Mamta, Mr. Baleshwar and Mr. Kamlesh for their assistance and valuable contributions.

I thank almighty God for showering his blessings always to provide me enormous patience, inspiration, faith and strength to carry out this research work.

(Mamta Chauhan)

TABLE OF CONTENTS

ABSTRACT

Lipases (triacylglycerol hydrolases) are an important group of hydrolases, having immense industrial applications in food, dairy, detergent and pharmaceutical sectors. Among hydrolases, lipases have gained special attention over few years owning to their ability to work in presence of extreme temperature, pH, organic solvents under non-aqueous conditions with chemo-, regio-, and enantio - selectivity. So by keeping in view the immense applications of lipase, the present work has been focused on production, characterization and purification of bacterial lipase and its evaluation as a potential detergent additive.

The bacterial strain, *Staphylococcus arlettae* JPBW-1 MTCC5589 was isolated from a rock salt mine Darang HP, India was identified as lipase producer. Higher lipase yield was observed when cultured in LB media (pH 8.0) supplemented with soybean oil (12 %) using 10 % inoculum for 3 h at 37 °C under submerged conditions. Partially purified lipase (60 % Ammonium sulphate) was found to be active over a broad range of temperature (30–90 °C), pH (7.0–12.0) and NaCl concentration (0–20 %). Enhanced lipase activity has been observed in presence of metal ions such as Mn^{2+}, Ca^{2+} and $Hg^{2+}$and activity inhibition with K^{+}, Co^{2+} and Fe^{2+}. Moreover, lipase retained its activity in presence of detergents (Triton X-100, Tween 80) and organic solvents (up to 30 % (v/v) of benzene, xylene, *n*-hexane, methanol, ethanol and toluene).

A modeling integrated optimization has been performed to model and optimize the lipase production through Response surface methodology (RSM) integrated Genetic algorithm (GA). For building the RSM model, three-level five-factorial central composite design was utilized by considering the individual and interaction effects of submerged fermentation variables on lipase production. The accuracy of the model was evaluated through significance test, ANOVA and R^2 value of 96.6 %. The validated input space of response surface model has been utilized for optimization through binary coded GA. In this study, tournament size of two, uniform crossover probability (Pc) of 0.5, mutational probability (Pm) of 0.0015, population size of 210, and maximum

number of generations of 815 were employed in search of optimal values for enhanced lipase production. An optimum lipase yield of 6.5 U/mL has been obtained using binary coded genetic algorithm predicted conditions of 9.39 % inoculum with the oil concentration of 10.285 % in 2.99 h using pH of 7.32 at 38.8 °C and validated through triplicate experimental runs.

Having good stability in presence of surfactants, salt conditions and organic solvents, the partially purified bacterial lipase was evaluated for its detergent ability in removing the olive oil stains from the cotton fabric. After initial stability studies of lipase with surfactants, commercial detergents and oxidizing agents (the usual detergent formulation ingredients), a set of presoak solutions was formulated so as to test the lipase in combination with detergent and buffer. Better olive oil removal (62 %) was obtained using 40 U lipase with 0.5 % wheel in 45 min at 40 °C from the soiled cotton fabric. Response surface methodology coupled with genetic algorithm has been utilized for modeling and optimization of bacterial lipase added washing process. An optimum value of 79.6 % oil removal was achieved with the GA-predicted process variables of 0.69 % detergent, 47.37 U of lipase, buffer pH of 7.2, and washing temperature of 37.18 °C in 26.11 min, which was 27 % more than the oil removal without lipase.

A three step purification (Ammonium sulphate precipitation, Ion exchange and Gel filtration chromatography) strategy was utilized for purification of bacterial lipase and achieved 27 fold purification with specific activity of 32.5 U/mg. The molecular mass of 45 kDa approximately was estimated through PAGE studies. An amino acid sequence which showed similarity with α/β hydrolase fold gi|427702968 *Cyanobium gracile* PCC 6307 was identified through MALDI –TOF coupled homology modelling.

The overall results of this study helps to introduce a new extremophilic bacterial lipase to enzyme industry and for developing a bacterial lipase based detergent formulation. Moreover, the present work also demonstrated the feasibility of statistical design tools integration with computational tools for modeling and optimization of process conditions for achieving enhanced results.

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS & ABBREVIATIONS

°C	Dergee Celsius
%	Percentage
µM	Micromolar
16S RNA	16S Ribosomal RNA
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization- Time of Flight
CM	Carboxymethyl
DEAE	Diethylaminoethyl Cellulose
DMSO	Dimethyl Sulfoxide
DOE	Design of Experiment
EC	Enzyme Classification
EDTA	Ethylenediaminetetraacetic acid
GA	Genetic Algorithm
GC	Gas Chromatography
h	Hours
HPLC	High Performance Liquid Chromatography
IUBMB	International Union of Biochemistry and Molecular Biology
IUPAC	International Union for Pure and Applied Chemistry
KDa	Kilo Dalton
Km	Michaelis Menton Constant

L	Liter
LB	Luria Bertani
mg	Milligram
mM	Mill molar
MTCC	Microbial Type Culture Collection
NMR	Nuclear Magnetic Resonance
O.D	Optical Density
p- NPP	p-Nitrophenyl Palmitate
PAGE	Polyacrylamaide Gel Electrophoresis
PHB	Polyhyroxybutyrate
PMSF	Phenylmethylsulfonyl Fluoride
RPM	Revolution Per Minute
RSM	Response Surface Methodology
SDS	Sodium Dodecyl Sulphate
Smf	Submerged Fermentation
SSF	Solid State Fermentation
TLC	Thin Layer Chromatography
U/ml	Unit Per milliliter
Vmax	Maximum Reaction Rate

CHAPTER 1

Introduction & Review of Literature

1.1 Enzymes as biocatalysts

Bio-catalysis is a key technology to provide realistic solutions to many environmental issues and to promote sustainability, environment, energy, health and quality of life. As with the application of more selective and more accelerated reaction pathways can be worked out and formation of wastes can be avoided. In biocatalytic reactions, small managed quantities of biocatalysts are sufficient to accelerate reaction rate by lowering the activation energy of a reaction compared to the corresponding uncatalyzed reaction at the same temperature. Biocatalyst performs reactions or processes more efficiently under milder conditions (Bailey and Ollis, 1986). Biocatalyst is not consumed by the reaction itself so remained unchanged at the end of the reaction unlike other reagents that participate in the chemical reaction. Chemical reactions are far too slow to be effective under normal living systems conditions such as aqueous environments with neutral pH and temperature between 20 - 40 °C. In comparison, enzymes (biocatalysts) can achieve up to 10^7 fold faster reaction rates than the catalysts developed by chemical industry (Dixon and Webb, 1979). The acceleration in reaction rate is achieved by lowering the activation energy of the overall process as shown schematically in Fig 1.1.

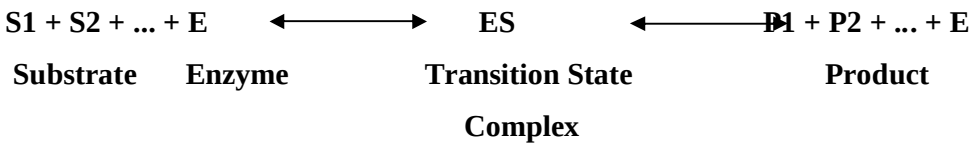


Figure1.1: Enzyme catalyzed reaction

As catalysts the enzymes alter the rate at which thermodynamic equilibrium is reached, but do not change the equilibrium. This implies that enzymes work reversibly. The reason why synthetic chemists have become interested in biocatalysis is mainly due to the need to synthesize enantiopure compounds as chiral building blocks for drugs and agrochemicals. Another important advantage of biocatalysts are that they are environmentally acceptable, being completely degraded in the environment.

Furthermore, the enzymes exhibit maximum activity under mild conditions, which minimizes problems of undesired side-reactions like decomposition, isomerization, racemization and rearrangement. Biocatalysts can be purified and separated with high yield under mild reaction conditions, which is also energy efficient and economic (Dunhaupt et al., 1991).

1.2 Advantages and disadvantages of biocatalysis vs. chemical catalysis

One of the unique characteristics of biocatalysts over conventional catalysts is its high selectivity. This selectivity is often chiral (stereoselectivity), positional (regioselectivity) and functional group specific (chemoselectivity). Generally, biocatalysts showed higher selectivity which is desired in chemical reaction and other benefits such as reduce or no use of protecting groups, minimized side reactions, easier to separate and environmental friendly (Brockerhoff and Jensen, 1974). Other unique features such as high catalytic efficiency and mild operational conditions are also very desirable in commercial applications. The most serious drawbacks of biocatalysts includes features of limited operating regions, substrate or product inhibition and reactions in aqueous solutions. However it was turn out about these drawbacks to be microconceptions. For example, many commercially available enzymes show excellent stability under process conditions. In addition there is an enzyme-catalyzed reaction, equivalent to almost every known organic reaction. Many enzymes can accept non-natural substrates and convert them into desired products. Almost all of the biocatalyst features can be tailored or rationally designed to meet the desired process conditions (Johannes et al., 2006).

1.3 Enzyme nomenclature

With the great progress achieved in the area of biochemistry in the 1950s' a large number of enzymes could be isolated and characterized. The International Union of Biochemistry and Molecular Biology (IUBMB) in consultation with International Union for Pure and Applied Chemistry (IUPAC) set up an Enzyme Commission (EC) to be in charge of guiding the naming and establishing a systematic classification for enzymes

(Liese et al., 2006). According to the type of reaction catalyzed, the Enzyme Commission has listed the enzymes into 6 main classes:

[a] Oxidoreductases (E.C.1…) catalyze oxidation/reduction reactions, transferring hydrogen, oxygen and/or electrons between molecules. In this important class belong dehydrogenases, oxidases, oxygenases and peroxidases.

[b] Transferases (E.C.2…) catalyze the transfer of groups of atoms [amino-, acetyl-, phosphoryl-, glycosyl-etc.] from a donor to a suitable acceptor.

[c] Hydrolases (E.C.3…) catalyze the hydrolytic cleavage of bonds. Many commercially important enzymes belong to this class, e.g. proteases, amylases, acylases, lipases and esterases.

[d] Lyases (E.C.4…) catalyze the non-hydrolytic cleavage of for example C=C, C=O, C=N bonds by elimination reactions leaving double bonds or reverse adding groups to a double bond.

[e] Isomerases (E.C.5…) catalyze isomerization and transfer reaction within one molecule. The most prominent member of this group is glucose isomerase or e.g. *Z-E* and cis-trans isomerization.

[f] Ligases (E.C.6…) catalyze the joining of two large molecules by forming a new chemical bond, usually with accompanying hydrolysis of a small chemical group, e.g., catalyze joining of C-O, C-S, C-N, etc. Ligases find limited applications only for synthetic purposes. The main classes are further subdivided into subclasses and subgroups.

1.4 Enzyme structure and function

All enzymes are proteins, with the exception of the recently discovered ribozymes. Proteins are linear polymers defined by the amino acid sequence (primary structure) linked by peptide bonds. To generate a specific surface as part of the active centre of an enzyme the protein chain has to be folded. Two structural arrangements of polypeptide become energetically favoured, the α–helix and β–pleated sheet that are further stabilized by H-bonds between the peptide backbones. Helices and pleated sheets are commonly found in proteins called secondary structure and when these secondary

structure elements are connected by loops to build a domain or a subunit, this level of organization is the tertiary structure, when more subunits are connected into homo or hetero-oligomers is called quaternary structure. The folded structure of a protein is stabilized by a network of non-covalent interactions such as hydrogen-bonds, disulfide bonds, hydrophobic interactions, ionic bonds, salt bridges and vander Waals interactions. The chemical potential of side chains found in amino acids is limited, there are no efficient electron acceptors, therefore, and it requires additional chemical potential by specific metal ions (Zn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}). Besides metal ions cofactors or coenzymes serve to activate groups and participate in the catalytic process. Cofactors (sometimes called prosthetic groups) are covalently bound to the protein and may undergo cyclic reactions during the catalytic process but will return to the ground state at the end. Coenzymes are bound in association-dissociation equilibrium to enzymes and have to be present in sufficient concentration to obtain maximal enzymatic activity. Some are regenerated in the catalytic cycle while bound to the enzyme. In some cases, the enzymes do not need any additional cofactors to show full activity is called an apoenzyme, and the apoenzyme-cofactor complex is called a holoenzyme (Drauz and Waldmann, 2002).

1.5 Emerging class of microbes: Extremophiles and their Biomolecules

Extremophiles are a class of microorganisms that are inhabitants of the most extreme conditions on the earth. These conditions are natural to them, and they are well adapted to them. For example, a hyperthermophiles *Pyrolobus fumari* grow at 113 ^{o}C but unable to grow below 85 ^{o}C (Huber and Stetter, 1998).

These microorganisms can grow in diverse climatic conditions and are classified accordingly. They grow at high temperature (thermophiles), low temperature (Psychrophiles), high pressure (barophiles), low or high pH (acidophiles or alkaliphiles), high salt concentration (halophiles), etc. Extremozymes produced by these are of immense importance in industrial processes, for instance thermophilic extremozyme offers many advantages like high rate of reactions, lower diffusional restrictions, lower viscosity and fewer contaminations (Ahmed et al., 2010). Similarly,

extremozyme from alkaliphiles had applications in the detergent industry for detergent formulations (Mitra et al., 2010) and leather industry (Sharma et al., 2001).

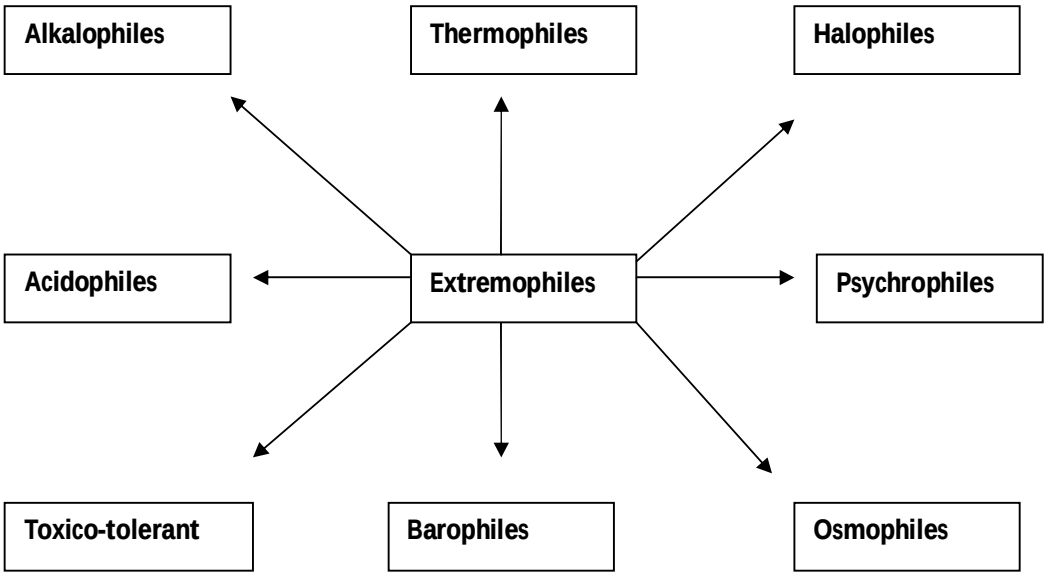


Figure1.2: Diagram showing different classes of extremophiles

Most of the industrial processes are carried out at high temperature, at different pH for a long period, using enzymes from microorganism of mesophilic origin. However, enzyme form theses microorganisms are not suited for industrial processes because of their low temperature and neutral pH ranges and non-reproducibility for long processes. This necessitates the isolation of extremophiles. Microorganisms of this class produce biomolecules called extremozymes that function well under extreme set of conditions (Sellek and Chaudhui, 1999).

However a new class of extremophiles called "solvent -tolerant," is attracting attention. This newly emerging class possesses the unique ability to grow in the presence of organic solvents (Gupta and khare, 2009). Microbes of this class possess generic adaptation mechanism to circumvent the toxic effect of solvents. For example degradation and transformation of hydrocarbon by *Rhodococcus* sp. leads to their solvent tolerance (Paje et al., 1997). These extremophiles devised various mechanisms, like, a shift in cell membrane fatty acids (Mohammad et al., 2006), transformation or degradation of the solvent (Paje et al., 1997) or by changing the fatty acid compositions

(Neilson et al., 2005). Also, there are many efflux pumps like tolC, mar, rob, saxS and acrAB on the membrane identified in *Pseudomonas* sp, which plays an important role in the excretion of solvent (Zhang and Poole, 1998). Biocatalysts from these extremophiles are studied from the perspective of non-aqueous enzymology. These biomolecules display striking novel properties and attain a high level of catalytic activity in organic solvents (Ogino and Ishikawa, 2001).

1. 6 Hydrolases

With the rapid technical developments in gene discovery, optimization, and characterization, enzymes have been increasingly used as biocatalysts. More than 75 % of industrial enzymes belongs to hydrolases but limited enzymes are commercially available and used successfully in industrial operations. Lipases at present are one of the most usable biocatalysts for biotechnological application at industrial level. Lipases, carbohydrases and proteases at present contribute less than 5 % and expected to rise 6.8 % annually by 2015 (Source: BCC Research). Microorganisms and their enzymes are used in a wide range of biotechnological activities such as synthesis of antibiotics, vitamins, or biopolymers, decontamination of soils, food, paper or textile industry; etc. To enhance the enzyme activity for industrial processes is one of the important aspect of bioprocess research, this is because enzyme catalyzed reactions are highly efficient, selective, environmental friendly and usually require mild conditions with less energy. Thus, there is an increasing interest for isolating new microbial enzymes and new enzyme-producing strains for their use in industrial conversions. Among these enzymes, lipases, esterases, cellulases, xylanases, pectinases, amylases and proteases are some of the most important (Gupta et al., 2004). However, lipase holds their place because of their capability to carryout reactions in aqueous and non-aqueous media and stability in the presence of organic solvents, in contrast to their counterparts. They bring about transformation of industrially important compound thus making its presence felt every now with a share of 5 % of enzyme market that is likely to grow in the near future.

Hydrolases catalyze the addition of water to a substrate by means of a nucleophilic substitution reaction. Hydrolases are the most commonly used biocatalysts in organic

synthesis. They have been used to produce intermediates for pharmaceuticals and pesticides. For the modification of fats and oils, cocoa butter equivalent synthesis, for biofuel and flavor enhancement. Among hydrolases there is particular interest towards amidases, proteases, esterases and lipases. These enzymes catalyze the hydrolysis and formation of ester and amide bonds. Lipases can hydrolyze triglycerides into fatty acids and glycerol. They have been used extensively to produce optically active alcohols, acids, esters and lactones by kinetic resolution. The natural function of most hydrolases in vivo is to hydrolyze natural compounds (e.g., acid derivatives to free acids). In organic solvents it is possible to run such reactions in the reverse direction, allowing for the synthesis of esters and amides. In most applications, biologically active compounds are needed in enatiomerically pure forms for use as drugs or agrochemicals. Therefore, the ability to prepare enantiomerically pure compound has become a key issue in organic chemistry. Being proteins built from naturally occurring chiral, enantiopure amino acids, hydrolases are also enantiomerically pure, chiral polymers. Thus when reacting with a chiral, racemic substrate, they will react faster with one enantiomer of the substrate than the other. This enantioselectivity is the basis of the widespread use of hydrolytic enzymes and so lipases.

Considering the biological relevance and variability of lipids, a great development has been made in biodegradation of lipids. Esterase (E.C. 3.1.X.X), which belong to group of hydrolase family capable of catalyzing the cleavage of chemical linkage by the addition of water molecules (Bornscheuer, 2002). Although esterases catalyses the hydrolysis of ester bonds of lipids and other compounds, they also able to hydrolyze non-ester bonds. Esterases are classified according to their preference for specific bond, moiety or substrates they use for catalysis. Lipases are carboxylic ester hydrolases which act on acylglycerol to liberate fatty acids and glycerol **(**Gupta et al., 2004).

As this work focuses on lipases that are hydrolases thus in the following paragraphs, the characteristics of this enzyme group will be discussed in details.

1.7 Lipases

Lipases (triacylglycerol acylhydrolases (EC 3.1.1.3) are ubiquitous in nature and produced by various plants, animals and microorganisms. Lipase belongs to a class of serine hydrolases which catalyze the hydrolysis of triglycerides to glycerol and free fatty acids (Sharma et al., 2001). Lipases represent the most versatile enzymes that display a broad spectrum of substrate specificity (Lopez, 2010). In addition, lipases catalyze the hydrolysis and transesterification of other esters as well as the synthesis of esters and exhibit enantioselective properties (Singh and Mukhopadhyay, 2012). Microbial lipases especially from bacteria and fungi represent the most widely used class of enzymes in biotechnological applications (Gupta et al., 2004).

1.7.1 Current status of Enzyme market and lipase share

Enzymes are important biomolecules which successfully replace inorganic catalyst owing to their attributes of their specificity, high rate of reaction, eco-friendly and biodegradable nature etc. During the past few years, there is a noticeable sustainable growth in enzyme market which forecasts the further enhancement in a near future.

The global market for industrial enzymes is estimated at $3.3 billion in 2010 and is expected to reach $4.4 billion in 2015. India has a marginal share in the global market for industrial enzymes which is estimated to be at about $ 3387. 30 million. On the basis of application, industrial enzymes could be divided into four major categories, i.e. detergent enzymes, technical enzymes, food enzymes and feed enzymes. The technical enzymes segment could further be divided into textile enzymes, leather enzymes, pulp and paper enzymes, fuel ethanol enzymes and others. The major enzymes used in industrial enzymes market are amylase, lipase, protease, ligase, phytase, cellulose, xylanase etc. The market segmentation for various areas of application shows that 34 % of market is for food and animal feed followed by detergent and cleansers (29 %). However the year on year growth registered by technical enzymes segment was highest in past 4-5 years due to dramatic growth of the fuel enzymes segment (Source: BCC Research).

Industrial enzymes operate in an oligopolistic market with the presence of three major suppliers, Novozymes, Genencor International Inc., and DSM N.V. Their main market

segments are food (e.g., dairy, baking, brewing, beverage), animal feed, and technical applications.

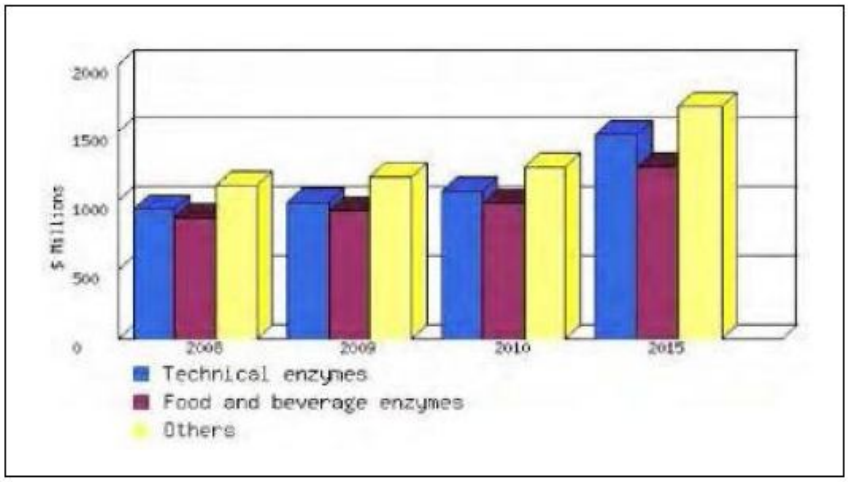


Figure 1.3: Global enzyme market scenario. (Source: BCC Research)

Novozymes is the largest supplier in food, feed and technical segments during 2010-2011, with an estimated market share of 47 % of the industrial enzyme market in 2010. Microbial lipases are of special interest because of their stability in organic solvents, lack of requirement for cofactors, their broad substrate specification and their high enantioselectivity. In 1994, NovoNordisk introduced the first recombinant commercial lipase Lipolase® which originated from the fungus *Thermomyces lanuginosus* and expressed in *Aspergillus oryzae*.

Table 1.1: Nova Nordisk in commercial Lipase production

Name	Action	Application
Lipopan®	Hydrolysis and Oxygen uptake	Baking Industry
Lipozyme®	Interesterification	Oil and fats industry
Novazym®27007	Hydrolysis	Pasta/noodles
Palatase	Hydrolysis	Dairy industry
Novozyme®871	Emulsification	Pet food industry

In 1995 Genencor International produced two bacterial lipases, Lumafats® from *Pseudomonas mendocina* and Lipomax® from *Pseudomonas alcaligenes*. Lipase produced by microorganisns like bacteria, fungus, and eukarya as well as by plants and animals is studied extensively. However, efforts are made to further to explore the application part of lipase and improve through directed evolution like techniques to enhance their efficiency and efficacy in industrial processes. Further, their commercialization is important which leads to further increase in share in enzyme markets.

1.7.2 Lipase producing microorganisms

Microbial lipases are more preferred than plants and animal lipases because of higher yield, regular supply irrespective of seasonal changes, easily handling and short generation time. Microbial lipases also work at neutral and alkaline pH optima and are often thermostable (Jaeger et al., 1999 and Joseph et al., 2007). The significance of microbial lipases in an industrial arena has already been greatly acknowledged with respective to their vast potential in numerous industrial applications (Hasan et al., 2006). Table 1.2 depicts the major bacterial sources for lipase production. Lipase production has been reported from a variety of bacteria, fungi and actinomycetes (Rapp and Backhus, 1992). However, the presence of lipases has been observed from 1901 A.D. for *Bacillus pyocyneus*, and *Bacillus fluorescens* (Jaeger et al., 1994) which represent some of today's best studied lipase producers, now named, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* respectively. Fungi and yeast like *Rhizopus nigricans, Aspergillus nidulans, Yarrowia lipolytica*, etc. (Pignede et al., 2000) have been reported for lipase production.

Lipase producers have been isolates from diverse regions range from soil or spoiled food material to extremophilic regions such as hot springs and rock salt mines. Lipases with novel properties have been studied from microorganism isolated from Antarctica Ocean (Feller et al., 1990), hot springs , high salt and sugar environment (Ghemen et al., 2000), compost heaps (Rathi et al., 2001).

Table 1.2: Lipase producing bacteria and actinomycetes

Bacteria / Actinomycetes	Reference
Achromobacter lipolyticum	Scholefield et al., 1978
Acinetobacter baumannii,	Hostacka, 2000
A. calcoaceticus 69-V,	Haferburg and Kleber, 1983
Acenetobactor haemolyticusi TA106	Jagtap et al., 2010
Aeromonas hydrophila MCC-2	Chuang et al., 1997
Alteromonas	Saimoku et al. 1999
Bacillus acidocaldarius,	Manco et al., 1998
B. alcalophilus,	Ghanem et al., 2000
B. atrophaeus SB-2,	Bradoo et al., 1999
B. thermoleovorans ID-1	Cho, 2000
Brevibacterium linens	Adamitsch and Hampel, 2000
Burkholderia cepacia	Ishii, 2001
Bacillus subtilis EH 37	Ahmed et al., 2010
Bacillus sp strain 42	Eltaweel et al., 2005
B. alcaloplilus	Ghanem et al.,2000
Chromobacterium viscosum,	Horiuti and Imamura, 1977
Lactobacillus casei-subsp-*casei* LLG,	Lee and Lee, 1990
Micrococcus varians CAS4	Zahran, 1998
Moraxella TA144	Feller et al., 1990
Proteus vulgaris K80	Kim and Oh, 1998
Pseudomonas aeruginosa,	Jaeger and Winkler, 1984
P. aeruginosa PseA	Gaur et al., 2008
Pseudomonas strain	Gao et al., 2000
Selenomonas lipolytica	Dighe et al., 1998
Streptococcus cremoris, S. lactis,	Kamaly et al.,1990
Yersinia	Kuznetsov and Bagryantsev, 1992

Lipase producer also isolated from desert soil, thermal station soil, oil mill, etc. (Ramdhane et al., 2011; Bayoumi et al., 2007; Eltaweel et al., 2005). However due to applications of lipases in organic synthesis, isolation of microorganisms which survive in the presence of toxic solvent is the current focus of research. The first report on the isolation of solvent-tolerant bacterial strain was put forward by Inoue and Horikoshi in

1989 for *Pseudomonas putida* (Gupta and Khare, 2009). The isolation of solvent tolerant microbes has been reported from ecological niches such as soil (Huertus and Dugue, 1998), deep sea (Kato et al., 1996) and are identified as belonging to genera *Pseudomonas* (Gupta et al., 2006), *Bacillus* (Bustard et al., 2002), *Rhodococcus* (Paje et al., 1997) and *enterobacter* (Gupta et al., 2006). In some cases, non-solvent tolerant strains are mutated to obtain tolerant strain such as utilization of spontaneous mutation of *E. coli* K-12 (Aono et al., 1991) to obtain some organic solvent-tolerant mutants. Komatsu et al., 1994 isolated *Pseudomonas aeruginosa* in n-hexane and p-xylene tolerant strain through repetitive subculturing.

Most of the bacterial isolates for lipase productions grow at various pH and temperature but generally prefer neutral pH (Gao et al., 2000 and Ghanem et al., 2000). However, there are also reports on alkaliphilic bacteria. On the contrary, fungus prefers acidic conditions for growth and lipase production (Pokorny et al., 1994). Beside these psychrophiles and thermophilic bacterial isolates, the microorganisms having different oxygen demand (Aerobic, microaerophilic and anaerobic), are also reported to produce lipase.

1.8 Microbial lipase production through Smf

Bacterial lipases, like most other types of industrial enzymes, can be produced either by submerged fermentation (SmF) or solid-state fermentation (SSF). In SmF, the nutrients and microorganisms are both submerged in water and nutrients are uniformly distributed (Singhania et al., 2009). Solid-state fermentation (SSF) is a process that involves solid matrix and occurs in the absence of any fluid in the space between substrate particles. Lipase production through submerged fermentation is dependent upon a number of factors including carbon and nitrogen sources, pH, temperature, aeration and inoculum size. Besides, Aguilar et al., 2004 reported that the expression of proteins may differ in SSF and SmF. Industrially important enzymes have traditionally been obtained from submerged fermentation (SmF) because of the ease of handling and greater control of environmental factors such as temperature and pH. SSF has some limitations, such as the limited choice of microorganisms capable of growth under

reduced moisture conditions, as well as the control and monitoring of parameters such as temperature, pH, humidity and air flow (Nahara et al., 1982 and Lonsane et al., 1985).

Bacterial lipases are mostly inducible enzymes, requiring some oil, fatty acids or fatty acid esters for induction. Lipases are mostly secreted extracellularly into the production medium during growth, but there are also reports on intracellular lipase production (Lee and Lee, 1989). Lipase production is organism oriented, but generally enzyme is activated and released during late logarithmic or stationary phase (Matselis and Roussis, 1992). The fast growing microorganisms tends to secretes biocatalyst within 12-24 hours (Stuer et al., 1986). Despite of the fact that lipase production is extensively studied, but it still requires a systematic approach to select the best production conditions for fermentation owing to their important effect on final yield. The earlier studies also highlighted the role of various environmental factors such as temperature, pH, nitrogen, carbon and lipid sources, agitation, and incubation period on lipase production.

Lipase production has been studied from various microorganisms using different substrates as inducer and their effect have been studied from time to time. Saod et al., 2005 studied the effect of different oils on lipase production. In their study they use olive oil, corn seed oil and sunflower oil as inducer and carbon substrate for lipase production for *Fusarium oxysporium*. Soybean oil was demonstrated an effective inducer for the production of fungal enzymes (lipases) that degrade plant seed oils (Messias et al., 2009). However, Pogaku et al., 2010 reported olive oil as best inducer for lipase production from *Staphylococcus sp*. Lp12 when compared with ground nut oil, coconut, castor oil. The importance of oil in lipase induction was anlaysed by Fadiloglu and Erkmen, 2002. Medium was supplemented with and without olive oil and it has been found that medium containing olive oil yield more lipase (5.5U/ml).

Lipase production has been studied using carbon sources like beef tallow, wool-sour effluent, whey, n-hexadecane and tween (Fonchy et al., 1999 and Chen et al., 1998). Jansen et al., 1996 describes the production of thermostable lipase from *Bacillus sp*.

strain Wai 28A45 in tripalmitin as a carbon source at 70 ^{o}C. A comparative study conducted by Gao and Breuil, 1995 to evaluate the effect of different oils like olive oil, soybean oil, sunflower, sesame, corn, peanut and seed cotton oil on lipase production from fungus *Ophiosstoma piceae*, maximum activity was seen with olive oil as inducer. However, high concentration of oil is inhibitory to enzyme production. However, further increment in concentration decreases the yield of biocatalyst significantly. The production of alkaline thermostable lipase from *Bacillus sterothermophilus* cited by Kim and Oh, 1998 in I L medium containing beef tallow and palm oil. Gerritse et al., 1998 reported alkaline lipase from *Pseudomonas aeruginosa* M-1 in medium supplemented with citric acid and soybean oil and inducer. Thermoalkalophiles which were capable of growing in pH 9 and at temperature 55 ^{o}C isolated by Bayoumi et al., 2007. Bacterial lipases have a neutral or alkaline optimum pH with the exception of lipase from *P. fluorescens* SIK W1 that had an acidic optimum pH 4.8. However, lipases from *Bacillus stearothermophilus* SB-1, *B. atrophaeus* SB-2 and *B. licheniformis* SB-3 are active over a broad pH range 3-12. Bacterial lipases generally have temperature optima in the range of 30-60 °C. For *B. licheniformis* MTCC-10498 maximum lipase production was observed at pH 7.5 (~0.4 U/ml). However, bacterial lipases with optima in both lower and higher ranges have been reported. Thermal stability data are available only for species of *Bacillus*, *Chromobacterium*, *Pseudomonas* and *Staphylococcus*. Eltaweel et al., 2005 studied the effect of various carbon sources like sorbitol, fructose and arabinose did not improve the lipase activity whereas addition of starch, rhamnose, melibiose and myo-inositole decreased the lipase production on lipase production from *Bacillus sp* stain 42. Sztajer and Maliszewsa, 1998 reported the enhanced lipase production in medium when supplemented with 5 % peptone, corn steep liquor and soybean meal for *Penicillum citrinum.* A thermostable lipase from *Pseudomonas aeruginosa* Kwi-56 was produced in medium containing 0.2 % peptone and 0.1 % yeast extract at 60 ^{o}C after 24 hrs (Izumi et al., 1990). *Bacillus strain* A30-1 (ATCC 53841) was reported for lipase production using 0.1 % yeast extract and 1 % ammonium chloride at pH 9.5 and 60 ^{o}C (Wang et al., 1995). An ammonium dihydrogen phosphate has the best nitrogen source for lipase production

from *Pseudomonas fluorescence* NS2W, which was found almost threefold more compared to the basal medium. However, lipase production was very low in medium soybean meal and corn steep liquor (Kulkarni and Gadre, 2002). A tryptone and yeast extract in combined form in 0.6 % and 0.2 % concentration as the best nitrogen source for lipase production from *Bacillus sp* strain 42 reported by Eltaweel et al., 2005. Along with carbon and nitrogen sources metal ions plays an important role in lipase production. Metal ions although required in millimolar concentration for microorganisms but influencing lipase production significantly. Kok et al., 1995 reported enhanced extracellular lipase production when medium was supplemented with Mg^{2+}, Ca^{2+}, Cu^{2+}, Co^{2+} from *Acinetobacter colcoaceticus* BD413. *Bacillus* species found to produce 7-fold increase in lipase production when growth medium was supplemented with Mg, Fe, and Ca ions (Janseen et al., 1994). The metal ions effected the lipase activity of *Pseudomonas pseudoalcaligenes* KKA-5 which retained activity in Cu^{2+} and Mg^{2+} but slightly inhibited by the presence of Mn^{2+}, Cd^{2+} and Cu^{2+} in the production medium (Sharon et al., 1999).

1.9 Modeling and Optimization of production parameters

Modeling and optimization are two of the most vital steps in the fermentation process for maximizing the efficacy of the process. RSM is a statistical technique which defines the effect of the independent variables (alone or in combination) on the entire processes and generates a mathematical model in addition to analyzing the effects of the independent variables of the process (Burkert et al., 2004; Kalil et al., 2000; Rao et al., 2000; Puri et al., 2002). This technique gives contours plots from linear, interaction and quadratic effects of two or more parameters and fits the experimental data to calculate the optimal response of the system. RSM can reveal the correlations between the factors and responses as well as the optimum level of each factor employed. There are several reports on optimization of lipase production by RSM (Khoramnia et al., 2011). The principle of RSM was described by Khuri and Cornell, 1996. Using RSM, the relationship among the variables, i.e. initial pH, temperature, the incubation period, inoculum volume, substrate volume were expressed mathematically in the form of a

polynomial model, which gave the response as a function of relevant variables. Modelling of biotechnological processes is a common tool in process technology. Several researchers acknowledged the Modeling efficiency of RSM for different industrial enzyme production along with the bacterial lipases production from *Bacillus* and *Burkholderia sp.* (Kumar et al., 2011). Response surface approach was used to find the optimum influential parameters of sunflower oil and incubation period at 1 % and 96 h, respectively (Kaushik et al., 2006).

GA is a search and optimization technique developed by Holland, 1992, which mimics the principle of natural evolution exploring the search space by incorporating a set of candidate solutions in parallel. The GA is highly relevant for industrial applications, because they are capable of handling problems with non-linear constraints, multiple objectives, and dynamic components– properties that frequently appear in the real-world problems (Goldberg, 2006). GA optimization is described as the simulation performed aiming to maximize a certain process objective. The search for the desired optimum is usually done using mathematical algorithms. Generally in case of optimization, the problem of interest must be formulated as a mathematical model which describes the system and its performance. The simulation of the process with a mathematical model facilitates the process optimization against highly expensive experiments, predicting process results for any set of decision variables. The simplicity, robustness, and higher convergence rates in lesser computational time account for their popularity in solving the complex, nonlinear problems. These algorithms differ with traditional and gradient based approaches, in searching a population of points in parallel not just a single point and utilizing the probabilistic transition rules instead of deterministic ones. In GA, the optimization search proceeds through three operators, namely, reproduction, crossover, and mutation (Holland, 1992). The reproduction (selection) operator selects good strings in a population and forms mating pool. The chromosomes are copied based on their fitness value. No new strings are produced in this operation. Crossover operation generates a child chromosome by exchanging some portion of the strings (chosen randomly) with string of another chromosome in the mating pool using a crossover probability (Pc). If the child chromosome is less fit than the parent chromosome, then it will slowly die in the subsequent generation. Mutation

was the last operation of GA optimization and used further to perturb the child vector using mutation probability (*Pm*).It alters the string locally to create a better string and to create a point in the neighborhood of the current point, thereby achieving a local search and maintaining the diversity in the population. The entire process is repeated till some termination criterion is met.The principal advantages of GA are domain independence, non-linearity and robustness. The only requirement for GA is the ability to calculate the measure of performance which may be highly complicated and non-linear. The above two characteristics of GA assume that GA is inherently robust. A GA has a number of advantages such as it can work with highly non-linear functions and suitable for parallel implementation. It can quickly scan a vast solution set. GA approach has been used to optimize the production of polyol, the production of xylitol (Patil et al., 2002), and a culture medium for fed-batch culture of insect cells. The successful utilization of RSM integration with GA for enzyme production has been acknowledged in a case of lipase production from *Staphylococcus xylosus* (Khoramnia et al., 2010) and *Geobacillus sp*. strain ARM (Ebrahimpour et al., 2008).

1.10 Lipase assay methods

There are a number of assays available for determination of lipase activity reviewed in the literature (Beisson et al., 2000 and Jaeger et al., 1994). Most of the methods are devised to estimate the products of hydrolytic reactions. Table 1.3 depicting the different assay methods practiced for estimation of lipase activity.

More advancement has been made for lipolytic activity determination through spectroscopic methods. Rollof et al., 1984 devised assay methods which involve direct turbidometric estimation of residual lipids, after reaction of lipids with substrates in emulsion. Similarly Robinson et al., 1989 developed a turbidometeric method for lipase activity. Besides these a few methods are developed where the color released after the hydrolysis of triglycerides used as a substrate is measured. Van Autryve et al., 1991 used rhodamine 6G for complexation with free fatty acids liberated during the reaction. The pink color was monitored at 513 nm. However, Medcova et al., 1981 assayed the monoglyceride lipase activity using tween 20 as substrate. In this case, lauric acid released was converted to copper laureate and measured at 435nm. Safarik, 1991

developed a method where substrate is immobilized and fatty releases were extracted with benzene and converted to their corresponding Cu [II] salt and measured spectrophotometrically.

There are enzymatic assays reported based on estimation of either release of glycerol or fatty acids as a consequence of biocatalysis by lipase on triacylglycerol. Wollett et al., 1984 reported the methods for estimation of released fatty acids. The quantity of fatty acid present in the medium was measured before and after incubation through spectrophotometrically. Fossati et al., 1992 described a colorimetric method for assaying lipase in serum by employing long-chain 1, 2,-diglyceride fatty acid as substrate, where glycerol released was assayed by a sequence of enzymatic reactions. Besides this in some spectrophotometric method, certain synthetic substrate are also used which upon hydrolysis imparts colour. For example β-Naphthyl Capryplate was used for assayed lipase in skim milk. The product β-Naphthol formed after catalysis was reacted with fast blue BB and colored product was then extracted in ethanol and measured spectrophotometrically (McKellar, 1986). Kurooka et al., 1977 described an assay using 2, 3-dimercaptopropan-1-ol tributyrate as substrate and 5, 5' dithiobis (2-nitro-benzoic acid) as chromogenic reagent. Richardson et al., 1989 used substituted arylethene derivatives as substrate and the products of these compounds are coloured, making them suitable precursors for chromogenic enzyme substrates. Para- nitrophenyl-esters of various chain length fatty acids are also used as substrates (Winkler and Stuckmann, 1979). However, these compounds are not suitable for specific lipase assays because they are cleaved by esterase also (Stuer et al., 1986). Some spectrophotometer assay can be performed in the presence of organic solvent that is quite useful during lipase purification with reverse micelle methods (Aires- Barros and Cabral, 1991). Rogel et al., 1989 employed spectrophotometer property of some natural occurring fatty acids like cis- parinaric for lipase activity estimation.

Table 1.3: Overview of lipase assays

Spectrophotometry					
Substrate	**Product**	**Method**	**Final Product**	**Wavelength in nm**	**Ref.**
2,3-dimercaptopropan-1-ol Tributyrate	Glycerol analogue [2over 3 positions]	Reaction with DTNB	TNB	412	Kurooka et al., 1977
T	*p*-nitrophenol	Product is colored		410	Winkler and Stuckmann 1979
Glycerides [triolein]	Free fatty Acid	Enzymatic Conversion	NAD	340	Woollett et al., 1984
Aryleethene Derivatives	**Hydrolysis products are colored**			Variable	Richardson et al., 1989
Glycerides	Free fattyAcid	Complex Formation	Rhodamine 6G	513	van Autryve et al, 1991
Glycerides	Free fatty Acid	Complex Formation	Cu [II] salt	715	Safarik 1991
1-2- diglycerides	Glycerol	Enzymatic Conversion	Quinine	550	Fossati et al., 1992
		Fluorescence			
Glycerides containing pyrene ring	Free acid analogues or aggregated substrate	Fluorescence Shift	Free acid analogues or glyceride analogues	340, 400, 450	Thuren et al., 1987
Glycerides	Free fatty Acid	Complex Formation	11[dansylamino] undecanoic Acid	350, 500	Wilton, 1990 & 1991

1.11 Purification and characterization of lipase

Enzyme purification is a series of processes intended to isolate a single type of enzyme from a complex mixture. Wide ranges of techniques are available and the choice depends on factors such as nature of the source i.e., extracellular or intracellular, scale of operation, stability of the enzyme etc. The extracellular products mainly suffer from dilution problem where the level of contaminants are considerably less as the selective secretion of the products takes place through the cell wall barriers. The level of the contamination is more for the intracellular products, where the dilution problem is not

predominating because of the other intracellular products being released upon disruption of the cell. Purification methods are employed in order to understand enzyme functions, structure and its relationship with an environment. Purification of lipase normally involves steps that depend upon the purity desired. Many lipases are purified, characterized and crystallized (Sharma et al., 2001). In a case of extracellular lipases, the first step is the removal of cells by ultracentrifugation or centrifugation, but in case of intracellular lipases an additional step of cell lysis is required. The extracellular enzymes suffer from dilution problem to concentrate the desired extracellular protein precipitation can be attempted. Ammonium sulphate or any other suitable organic solvent can be used as the precipitating agent. After complete precipitation the fraction has to be centrifuged to separate the precipitated protein. Precipitation often gives a high average yield (Aires-Barros et al., 1994) although with limited purification, and such enzyme preparations are apt for use in detergent formulations. A single chromatographic step may be not enough for getting the level of required purity and combination of these techniques should be applied. Ion exchange chromatography followed by gel filtration is most used techniques in lipase purification protocols (Saxena et al., 2003). Ion exchange chromatography is the most common chromatographic method; used in 67 % of the purification schemes analysed and in 29 % of these procedures, it is used more than once. The most frequently employed ion-exchangers are the diethylaminoethyl (DEAE) group in anion exchange (58 %) and the carboxymethyl (CM) in cation exchange (20 %). Gel filtration is the second most frequently employed purification method, used in 60 % of the purification schemes and more than once in 22 % of them. In further step, chromatographic techniques like hydrophobic interaction chromatography are involved (Gupta et al., 2009). Borkar et al., 2009 reported the purification of extracellular lipases from *Pseudomonas aeruginosa* SRT 9 using ammonium sulphate (30 % - 90 %) followed by dialysis and then subjected to chromatographic column containing phenyl sepharose CL-4B and mono QHRS/S column. Purification of thermo-alkaliphilic lipases from *Bacillus licheniformis* B42 was reported by Bayoumi et al., 2007. Purification methods consist of ultrafiltration, followed by ammonium sulphate precipitation. Finally gel filtration

chromatography containing sephadex G200 and G100 column was done. The whole purification steps results in 27.33 purification fold. Ahemd et al., 2010 reported the partial purification of lipase from thermostable alkaline lipase. The lipase was partially purified through ammonium sulphate and purified using hydrophobic interaction chromatography resulting in 17.8 fold purification fold and 41.9 U/ml of specific activity. Since the importance of lipases from industrial application point of views necessities the critical evaluation of microorganisms isolated from solvent rich conditions. Lipase from such microorganisms has been purified by using combination of various chromatographic techniques. Gour et al., 2008 purified lipases from solvent tolerant strain *P. aeruginosa* the extract was subjected to ultrafiltration followed by lyophilization of retantate. Then crude powder of enzyme was dissolved in buffer and subjected to (sephadex G-100 column) gel filtration chromatography leading to 8.6 fold purification and 51.6 % recovery. Molecular weight of lipase was 60 kDa. The lipase enzyme was found to be stable at pH range of 6-8.5 and temperature range 25-50 ^{o}C. Demir and Tekul, 2010 reported the purification and characterization of lipase from photosynthetic cyanobacterium *Spirulina platensis.* The purification was performed using sequential protocol of precipitation with ammonium sulphate followed by dialysis, DEAE- sepharose anion exchange chromatography and sepharose-6B gel filtration chromatography with 375 purification fold and have 29.35 % final yield. The molecular weight of the protein was 45 KDa. The purified enzyme was further studied for hydrolytic activity which come optimum at temperature 45 ^{o}C and pH 6.5, values of K_m and V_{max} calculated from the Lineweaver–Burk plot using p-nitrophenyl palmitate (p-NPP) as hydrolysis substrate were 0.02 mM and 38.9 mol min−1 mg−1, respectively. Mohemed et al., 2011 reported the purification and characterization of lipase from *Mucor recemosus.* Cell free filtrate was concentrated by lyophilization followed by dialysis. Dialyzed material was subjected to chromatographic column DEAE-Sepharose column. Further fraction showing highest lipase activity using olive for hydrolysis was subjected to Sephacryl S-200 column. Molecular weight of the protein estimated by SDS –PAGE and found out to be 20 kDa. Vmax and Km for LII were estimated to be

55.5 µmol oleic acid/min/ml and 2 % olive oil, respectively. Optimum pH and temperature for purified enzyme were 5.0 and 40 ^{o}C respectively.

Interaction between an enzyme molecule and the surrounding water molecules are of crucial importance for optimum enzymatic activity and catalysis. The three dimensional structure which is resulted due to a balance between hydrogen bonds, hydrophobic effect, vander waals forces and dipole interaction are distorted when a biocatalyst is dissolved in organic medium. In anhydrous organic solvents, high enzyme stability has been observed but it comes with reduction in activity (Torres and Castro, 2004). The presence of solvent in the media however leads to increase the thermal stability of biocatalyst due to the absence of water which supposed to place enzyme in restrained conformation. Addition of small quantity of water however increases the enzyme activity since water conferred the flexibility to biocatalyst. Hydrophilic in contrast to their counterpart results in decreases in activity due their water stripping effect, thus placing biocatalyst in more constrained form. There is a correlation between enzyme activity and hydrophobicity (log P value). Generally solvent with log P value less 2 are not recommended better solvent whereas above 2 log P value solvent are better and support enzyme biotransformation. Many research groups evaluated the effect of organic solvent on lipase. Eltweel et al., 2005 studied the effect of different organic solvent having different log P value on *Bacillus sp* strain 42. Benzene and hexane slightly enhanced the lipase activity and found stable in toluene and xylene with residual activity of 84.7 % and 79.7 %. Similar effect of organic solvent benzene and hexane in 60 % (v/v) on *Bacillus* J33 was reported by Nawani et al., 1998. Hun et al., 2003 reported that n-hexane and p-xylene enhanced lipase activity of *Bacillus spaericus* 205 by 3.5 and 2.9 fold, respectively. Gaur et al., 2008 studied the effect of organic solvents on *P. aeruginosa* Pse in polar (log P <0.3) and non- polar solvent (log P value from 2.0- 7.0), stability was evaluated at 50 % concentration of organic solvent in n-hexane, heptane and DMSO. Lipase retained 94, 90 and 100 % of activity in these solvent respectively. Ahmed et al., 2010 reported effect of solvent on partial purified lipase from *B. subtillis* EH 37. The effect was studied on two concentrations at 15 % and 30 % in polar and non-polar solvents. Enzyme shows small decrease in the activity

in all solvent tried at both concentrations except hexane which in higher concentration (30 %) slightly enhanced the lipase activity.

Halophiles are organisms adapted to thrive in extreme conditions of salinity, distributed all over the world in hypersaline environments, which includes hypersaline brines in arid, coastal, and even deep sea locations, as well as in rock salt mines (Kushner, 1978). The intracellular machinery of these prokaryotes has evolved to function at very high salt concentrations. During recent years, these halophiles have been considered of great interest because of their biotechnological potential, notably for producing genes and enzymes of industrial interest and accumulating a variety of organic compounds, called compatible solutes, useful as enzymes or cell stabilizing agents. Moreover, microorganisms are themselves used in various biotechnological applications (e.g. bioremediation).

However, in spite of a growing interest in the use of halophilic enzymes for biotechnological applications, there are relatively few reports in the literature about their production and characterization (Bhatnagar, 2005). Halophilic microorganisms have greater capacity to produce salt and thermo tolerant enzymes like cellulases, amylases, proteases, lipases and xylanases (Govender et al., 2009).

1.12 Properties and mechanism action of microbial lipases

1.12.1 Difference between "true" lipases and carboxylesterases

Lipases are generic terms which encompass two groups of enzymes. "Carboxylesterases" and "true lipase". These two groups are characterized on the basis of their preferences for short and long chain substrates. Several criteria have been used to distinguish "true" lipases (TLs, EC 3.1.1.3), also known as triacylglycerol lipases, from carboxylesterases (CEs, EC 3.1.1.1), and also called esterases. True lipase shows substrate specificity for long chain acylglycerol ester whereas carboxylesterases acts on short-chain and relatively simple ester (Bornscheuer, 2002). Although no strict definition of long chain or short chain is available but esters with an acyl chain length of more than 10 carbon atoms are recommended substrates for true lipases, on the contrary acyl chain length less than 10 carbon atoms are considered as the substrates for

carboxylesterases (Jaeger et al., 1999 and Fojan et al., 2000). Irrespective of the above consideration of substrates it has been seen that the truest lipase are capable of hydrolyzing carboxylesterases substrate's and vice versa (Jaeger et al., 1999 and Fojan et al., 2000). The difference in substrate specificity are attribute of scissile acyl binding site of the enzymes, which have to fit completely the acyl chain in both size and charge. A steric conflicts with substrate would takes place owing to decreased size of binding site, whereas increase in size leave free space result in sub-optimal binding of substrates which further reduce the activity. Because of these attributes carboxylesterases have small acyl binding pocket whereas true lipases have long hydrophobic scissile fatty acyl binding sites (Pleiss et al., 1998). Substrate specificity is further directly linked with preference of carboxylesterases and true lipases which they show for hydrophobicity and physical state of their substrate. True lipase prefers highly hydrophobic substrates which remain insoluble and form aggregates (Fojan et al., 2000 and Bornscheuer, 2002). Therefore biocatalysis mediated by true lipase occurs at lipid interface (Jaeger et al., 1999) on the other hands carboxylesterases activity is high on more water soluble substrates and it depends on substrate concentration.

1.12.2 Properties of bacterial lipases

The features of bacterial lipases have been studied mainly due to the industrial or clinical interest of these enzymes. They are very diverse enzymes with a wide range of enzymatic properties, although some conclusions can be obtained by comparing them. These conclusions, mainly focused on TLs, are briefly summarized below. Generally, bacterial lipases have neutral or alkaline pH optima although there are some exceptions (Bornscheuer, 2002). They are active over a broad pH range (pH 4 -12), mainly in the case of lipases from the genus *Bacillus* (pH 3-12). Bacterial lipases generally have temperature optima in the range of 30–60 °C, and they show thermal stability up to 60 ºC, although lower and higher (mainly for thermophilic *Bacillus* lipases) ranges have been reported. Furthermore, the thermostability of lipases may be enhanced by the addition of stabilizers such as ethylene glycol or glycerol. In addition, lipases are generally stable in organic solvents, such as ethanol or acetone, with few exceptions of stimulation or inhibition (Gupta et al., 2004). Many bacterial lipases are active only

when they combine with cofactors such as metal ions or small molecule. Different lipases show different response to these metal ions and those ions which function as activators for certain lipases inhibit the activity of few others. Many metal- dependent/ metallo lipases have been reported and Ca^{2+} has been found to be exhibit a stimulatory effect in all those enzymes (Zhang et al., 2009). This effect could be attributed to the formation of insoluble ion salts of fatty acids during hydrolysis, thus avoiding the product inhibition. In contrast, some lipases are inhibited by the presence of calcium ions. Furthermore, heavy metals like Fe^{3+}, Co^{2+}, Ni^{2+}, Hg^{2+} and Sn^{2+} inhibit drastically lipase activity, whereas Zn^{2+} and Mg^{2+} are slight inhibitors.

1.12.3 Reactions catalyzed by lipases

Lipases catalyze a wide range of reactions, including hydrolysis, inter-esterification, transesterification, alcoholysis, acidolysis, esterification and aminolysis. Therefore, lipases, especially microbial lipases have many industrial applications (Mohammed et al., 2011). Lipase catalyze wide range of reactions as shown below, thus is highly used in industrial application.

Ester Hydrolysis

$R_1\text{-COO-}R_2 + H_2O \longrightarrow R_1\text{-OH} + R_2\text{-COOH}$

Ester Synthesis

$R_1\text{-OH} + R_2\text{-COOH} \longrightarrow R_1\text{-COO-}R_2 + H_2O$

Intramolecular esterification

$OH - R - COOH \longrightarrow R\text{-COO-}R$

Synthesis of estolides and other polymers

$R_1\text{-COOH} + HO\text{-}R_2\text{-COOH} \longrightarrow R_1\text{-COO-}R_2\text{-COOH}$

Interesterification

$R_1\text{-COO-}R_2 + R_3\text{-COO-}R_4 \longrightarrow R_1\text{-COO-}R_3 + R_3\text{-COO-}R_2$

Transesterification by acidolysis

$R_1\text{-COO-}R_2 + R_3\text{-COOH} \longrightarrow R_1\text{-COO-}R_3 + R_2\text{-COOH}$

Transesterification by alcoholysis

$R_1\text{-COO-}R_2 + R_3\text{-OH} \longrightarrow R_1\text{-COO-}R_3 + R_2\text{-OH}$

Transesterification by aminolysis

$R_1\text{-COO-}R_2 + R_3\text{-NH}_2 \longrightarrow R_1\text{-CONH-}R_3 + R_2\text{-OH}$

1.12.4 Catalytic Versatility of lipases

Lipases are very versatile enzyme in terms of biocatalysis. They are serine hydrolases which attracted attention because of their capability to carry out wide range of reactions. They catalyze the hydrolysis of acylglycerol and synthesis of esters from glycerol and long chain fatty acids (Rodriguez et al., 2009). Besides this, exchange of acyl group (Inter and transesterification) among different substrates or even among different chemical groups of the same compound is catalyzed by lipases.

1.12.5 Structure and catalytic mechanism of lipases

1.12.5.1 The fold and lid

Tremendous achievement has been made regarding the structure of lipases since last couple of decades using X-ray and NMR technology. Studies reveal that there is not much difference among the lipases and they possesses similar structural fold (Jaeger et al., 1999). The structure is named as α/β hydrolases fold, which consists of central mostly parallel β-sheet with two strands connected by α-antiparallel strands. The active site of Alfa/ Beta fold has three catalytic residues namely serine, cysteine or aspartate, a catalytic residues aspartate or glutamate and a histidine residue. Nucleophilic amino acid in lipases is serine whereas catalytic residues can either be aspartate or glutamate. However exception to this rule can be seen in case of *Streptomyces scabies* esterases where instead of triad a catalytic dyad is present. The nuleophilic serine residues are located in highly conserved Gly- Xaa -Ser, Xaa - Glu pentapeptides (Ollis et al., 1992). Lipases are characterized by a phenomenon designated as “interfacial activation” where biocatalyst is known to operate at the interfaces of two-phase systems (Mendes et al., 2012). Due to the low water solubility of fats and oils, interface is the key point where biocatalysis takes place. In addition to this interface also serves as appropriate site for modulating lipolysis for further study (Reis et al., 2009 and Reis et al., 2010).

1.12.5.2 Catalytic mechanism of lipase

Crystallographic analysis of inhibitor lipase complexes reveals the mechanism of ester hydrolysis or synthesis**.** It is constituted by the four steps represented in Fig. 1.4. During the first step, the substrate binds to the nucleophilic serine yielding a tetrahedral

intermediate stabilized by the catalytic His and Asp residues, the helix C, and the oxyanion hole. In the next step, alcohol is released and an acyl–enzyme complex is formed. Nucleophile attack (water in hydrolysis, alcohol or ester in trans or interesterification) forms again a tetrahedral intermediate, which yields the final product (an acid or an ester) and free enzyme (Bornscheuer, 2002). The detailed steps of lipase mechanism of action in hydrolysis reaction are summarized below:

Step 1: The lipid binding initiated the hydrolysis and activated the nucleophilic serine residue by the neighbouring active histidine, to which a proton from the serine hydroxyl group is transferred. Catalytic acid facilitates the proton transfer, which precisely orients the imidazole ring of the histidine and partly neutralizes the charge. After activation, the oxygen atom (O) of the serine hydroxyl group attack on the activated carbonyl carbon of the lipid ester bond (Jaeger et al., 1999).

Step 2: The formation of transient tetrahedral intermediate occured, which is characterized by a negative charge on the carbonyl oxygen atom of the scissile ester bond and four atoms bonded to the carbonyl carbon atom arranged as a tetrahedron. The intermediate is stabilized by the macrodipole of helix C, and by hydrogen bonds between the negatively charged carbonyl oxygen atom (oxyanion) and at least two main-chain NH groups (oxyanion hole).

Step 3: The histidine donated its additional proton to the ester oxygen of the susceptible bond, which is thus cleaved. At this stage, the acid component of the substrate is esterified to the nucleophilic serine (the covalent intermediate), whereas the alcohol component diffuses away (Jaeger et al., 1999).

Step 4: The additional proton of histidine is subsequently donated to the oxygen atom of the active serine residue, which breaks the ester bond between serine and the acyl component, and releases the acyl product. After diffusion of the acyl product, the enzyme is ready for another round of catalysis (Jaeger et al., 1999).

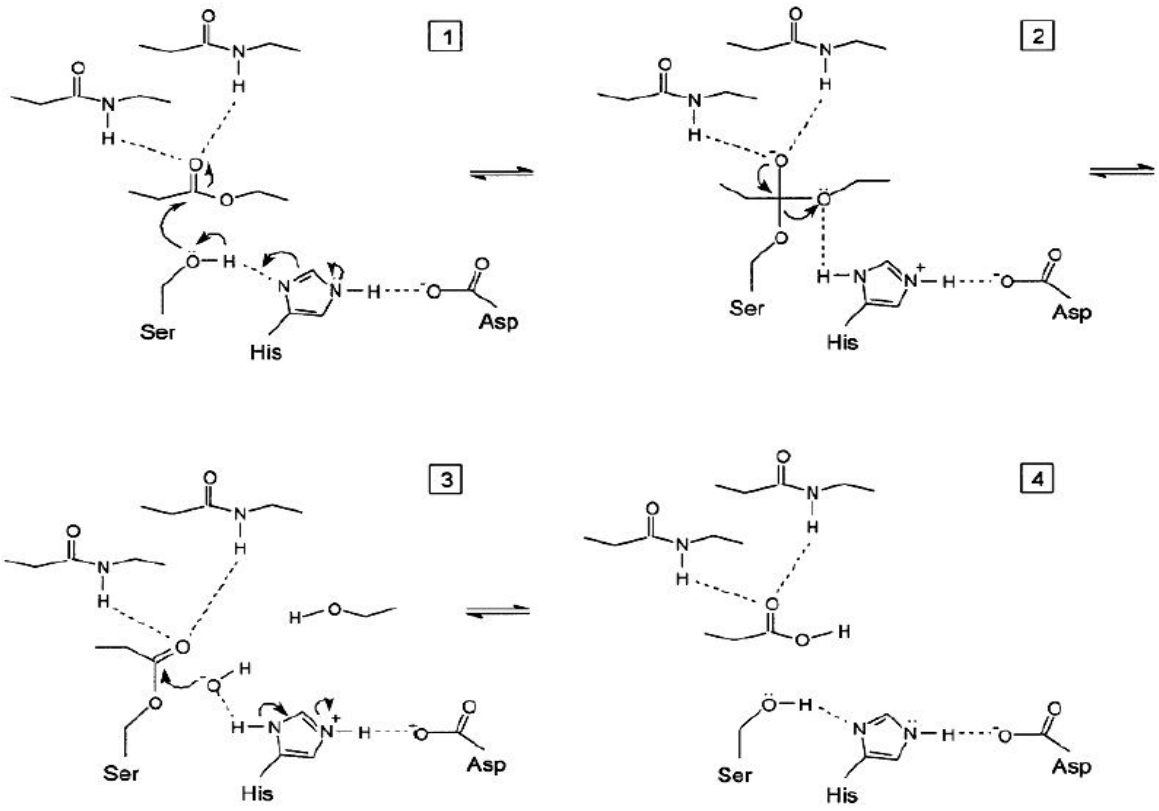


Figure 1.4: Mechanism of action of lipase in hydrolysis reaction

1.13 Industrial applications of lipases

Lipases are important hydrolytic enzymes with innumerable applications and indurtrial potential. Lipases are the most versatile biocatalysts and they bring about a range of bioconversion reactions such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis. These reactions used in food technology applications in fats and oil industry, dairy industry, pharmaceuticals and bakery industry provide a platform for lipase to act as a potential industrial enzyme. The alkaline thermophilic lipases find application in detergent industry. Table 1.4 enumerates a few of the most significant industrial applications of microbial lipases.

1.13.1 Lipases in fat and oleochemical industry

Some fats are more valuable than other because of their structure. Therefore less valuable fats are converted into more useful using blending of chemical methods but these tends to random products (Hasan et al., 2006). Therefore lipase catalyzed transesterification of cheaper oils can be used. For example, production of cocoa butter from palm mid fraction. Interesterification and hrdrogenation are techniques which have been useful in the preparation of glyceride products for their use in the manufacture of butter and magrine. Inter and transesterification mediated by lipase are used to increase the nutrional value of acylglycerised or also suitable for parental feeding.

1.13.2 Lipases in detergents

Lipases were generally added to the detergents primarily in combination with proteases and cellulases. However, other enzymes such as amylases, peroxidases and oxidases are also reported to be added in detergent preparations (Kottwitz et al., 1994) Removal of oil/fatty deposits by lipase are attractive owing to its suitability under milder washing conditions.

To be a suitable additive in detergents, lipases should be both thermostable as well as alkalophilic and capable of functioning in the presence of the various components of washing powder formulations (Jaeger et al., 1994). *Pseudomonas* lipase preparations have been used for preparation of washing powder formulations. *Pseudomonas medocina* (Lumafast) and *Pseudomonas alcaligenes* (Lipomax) lipases have been manufactured by Genencor international USA, as detergent additive (Jaeger et al., 1994 Jaeger and Reetz , 1998). The Novo group has reported a highly alkaline, positionally non-specific lipase, from a strain of *Streptomyces* sp. that was useful in laundry and dish-washing detergents as well as industrial cleaners (Pandey et al., 1999). Several lipase-producing organisms and their manufacturing processes are patented for preparation of detergent lipases (Lawler and Smith, 2000).

Table 1.4: Industrial applications of lipases

Source	Applications/ properties	Referances
A. calcoaceticus	Waste management -Heating oil/furnace oil, removal of fats, oils and greases	Mrin et al., 1995
Bacillus subtilis	Biomedical applications- Cephalosporin derivative	Usher et al.,1995
Bacillus sphaericuc 206Y	Produces solvent tolerant lipase	Hun et al.,2003
Staphylococcus haemolyticus	100% toluene, benzene and *p*- xylene tolerant	Neilson et al., 2005
Sphinobacterium mizutae	Mineralization of a high concentration of isopropanol	Mohammad et al., 2006
Cnadida antarctica lipase B	Biomedical application: alcoholytic resolution of [R,S]-flurbiprofenyl azolides for preparation of [R]-NO-flurbiprofen ester prodrugs	Ciou et al., 2011
Candida lipase Lipase B [Novozyme 435] and *Rhizomucor miehei* lipase [Lipozyme RM IM]	Application in Biomedical and food industry	Celiz and Daz, 2011
Chromobacterium viscosum	Biomedical applications- precursors of Vitamin D	Fernandez et al., 1995
Mucor racemosus	Biomedical application: treatment of cellulite	Mohemed et al., 2011
Pseudomonas cepacia	Biomedical applications- Syntheisis of Rapamycin-42	Adamezyk et al., 1994
Pseudomonas fluorescens	Biomedical applications- Hydantoins	Yokomatsu et al., 1995
Pseudomonas aeruginosa PseA	Produces solvent-tolerant lipase	Gaur et al.,2005; Gupta et al., 2008
Bacillus sp	Food and Processing industry	Ahmed et al., 2010
Candida Antarctica	Application in lipid transformation	Mugo and Ayton, 2010
Thermomyces lanuginosus	In organic synthesis	Cai et al.,2011

1.13.3 Lipase for ester synthesis

In the recent, the world has confined the twin crises of fossil fuel, depletion and environment degradation. The acute shortage and drastic hike in fuel price of petroleum and refined products derived from petroleum need to devise alternatives. Further the diesel engines emit relatively high level of certain pollutants especially particulate, further leads to global warming. All these big concern fueled extensive research effort

to replace some or all petroleum based diesel fuel with a clean- burning fuel derived from a renewable source such as from farm crops. However, pure form of these vegetable oils when fueled in diesel engines, they cause excessive engine wear and fuel injector choking with high smoke values. Also their viscosity is much higher than petroleum based diesel fuel. Therefore various attempts have been made to reduce the viscosity of vegetable oils either by dilution, microemulsion, by pyrolysis, by catalytic cracking down or by transesterification. All of these problems are overcome by transesterifying oil with methanol in the presence of acid or base as catalyst leads to formation of ester. However the problem further arises with chemical methods of ester synthesis. The industrial process for methyl esters production is carried out by using inorganic catalyst like NaOH, KOH but the major drawback is that the homogenous catalysts are removed with glycerol layers after reaction and cannot be reused. Formation of soap during reaction further leads to the low yield of ester formation and makes it difficult to separate glycerol and ester.

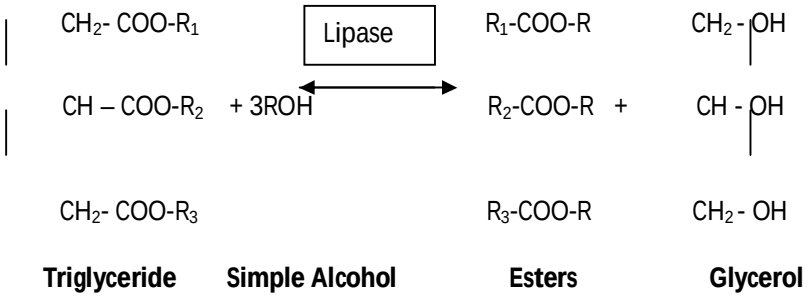


Figure 1.5: Transesterification of triglycerides with alcohol.

1.13.4 Lipases in leather industry

Lipases represent a more environmentally safe and sound method of fat removal. Degreasing is an essential stage in the processing of fatty raw materials such as small animal skins and hides from intensively fad cattle. Conventional methods using chemical are not desirable since they raise environmental problems such as volatile

organic compound emissions. However, lipase can remove fats and grease from skin and hides, therefore, both alkaline stable and acid active lipases are used in skin and hide degreasing (Hasan et al., 2006). Many *Bacillus sp*. which grew successfully under highly alkaline conditions, were found to be useful in leather processing (Haalck et al., 1992).

1.13.5 Lipases in biomedical application

Lipases may be used as digestive aids. Lipases are the activators of tumor necrosis factor and therefore can be used in the treatment of malignant tumors (Kato et al., 1989). Lipases from *Candida rugosa* have been used to synthesize lovastatin, a drug that lower serum cholesterol level. Lipase from *S. marcescens* was used for asymmetric hydrolysis of 3-phenlyglyceridic acid ester which is a key intermediate in the synthesis of diltiazem hydroclhorise a coronary vasodilator (Matsumae et al., 1993). Mohemed et al., 2011) reported the *Mucor racemosus* lipase for the treatment of cellulite.

1.13.6 Lipases in environmental application

Bioremediation for waste disposal is a new avenue in lipase biotechnology. Bouchon et al., 2000 reported the lipase application in wastewater treatment, bioremediation in fat contaminated cold environment and active compound synthesis in cold synthesis. Wakelin and Forster, 1997 investigated the microbial treatment of waste from fast-food restaurants for the removal of fats, oils and greases. They cultivated pure and mixed microbial flora known to produce lipases and other enzymes. *Acinetobacter* sp. was the most effective of the pure cultures, typically degrading 60–65 % of the fatty material.

1.13.7 Lipase in cosmetic industry

Unichem international (Spain) has launched the production of isopropyl myristate, isopropyl palmitate and 2- ethylhexylpalmitate for use as an emollient in personal care products auch as skin and sunscreen lotion (Hasan et al., 2006). Wax ester hace similar application in personal care products and are manufactured by enzymatically (Croda Universal Ltd.) using lipase from *C. cylindracea* in batch bioreactor. Retionoids (vitamin A and derivatives) with great potential in personal care product and prepared by lipase through immobilized lipase (Maugard et al., 2002).

1.13.8 Lipase in pulp and paper industry

The pulp and paper industry processes huge quantities of lignocellulosic biomass every year (Hasan et al., 2006). Historically, enzymes have been found some uses in paper industry, but their use manly confined to areas like modification of raw starch. Pitch or the hydrophobic component of wood causes severe problems in paper and pulp manufacturer (Jaeger and Reetz 1998). Lipases are used to short out this problem to remove pith from pulp for paper making (Sharma et al., 2001).

1.13.9 Specialized application of lipases in organic solvent

Lipases in addition to their natural function of hydrolyzing carboxylic ester bonds and also catalyze esterification, interesterification and transesterification reaction in organic medium (Hasan et al., 2006). Lipases have been used to catalyze region, chemo-, and stereo selective transformation of pharmaceutically or commercially important compounds in free or in immobilized form (Sharma et al., 2001 and Tufvesson et al., 2011). For industrial application cost and performance plays an important role for selecting a biocatalyst so enzyme immobilized for the synthesis of chemicals. Immobilizations improve stability, productivity as well as the scope of biocatalyst. Tufvesson et al., 2011 reported the immobilization of *Candida Antarctica* lipase B for the esterification and amidation reactions.

1.13.10 Lipases in resolution of recemic acids and alcohol

One of the attribute of lipase is steroslectivity which is used to resolve various recemic organic acids mixture in biphasic systems. Lee et al., 2004 reported the enantioselective resolution of (R) 3- hydroxyl butyric acid, (S)- mandelic acids and (3S,4R)-cis-3hydroxy-4 phenylazetidin 2 one from *E.coli* displaying lipase from *P. fluorescence* on the cell membrane with 99 % enantiomeric success. Resolution of 1-4 dihydropyridine the S-enatiomer of acrylopropionic acids (a non-steroidal antiinflamatory agent) has been accomplished through lipase mediated transesterification (Gotor, 2002). Similarly lipase mediated hydrolysis was used for resolution of profens (2-aryl propionic acids) (Lee et al., 1995). *Chromobacterium viscosum* lipase has also been studied for the regioselective acylation of a ring synthon of 1 α, 25-dihydrovitamin D_3.

1.14 Objectives

Having a special importance towards the extremophilic enzymes in industrial arena and multifold application status of bacterial lipases, the objectives of the present research work have been framed as follows:

1. Production of lipase by *Staphylococcus arlettae* JPBW-1 through SmF and its characterization.
2. Modeling and optimization studies of lipase production through statistical integrated artificial intelligence approach.
3. Evaluation of lipase for its detergent additive capability.
4. Modelling embedded optimization strategy for formulation of bacterial lipase based bio-detergent.
5. Purification of *Staphylococcus arlettae* lipase.

CHAPTER 2

Production of Lipase by *Staphylococcus arlettae* JPBW-1 through SmF and its Characterization

ABSTRACT

Studies on lipase production and characterization were carried out with a bacterial strain *Staphylococcus arlettae* JPBW-1 isolated from rock salt mine, Darang, India. Higher lipase activity has been obtained using 10 % inoculum with 12 % of soybean oil as a carbon source utilizing a pH 8.0 in 3 h at 35 ℃ and 100 rpm through submerged fermentation. Partially purified *S. arlettae* lipase has been found to be active over broad range of temperature (30-90 °C), pH (7.0-12.0) and NaCl concentration (0 to 20 %). It has been shown extreme stability with solvents such as benzene, xylene, n-hexane, methanol, ethanol and toluene up to 30 % (v/v). The lipase activity has been found to be inhibited by metal ions of K^+, Co^{2+} and Fe^{2+} and stimulated by Mn^{2+}, Ca^{2+} and Hg^{2+} metal ions. Lipase activity has been diminished with denaturants, but enhanced effect has been observed with surfactants, such as Tween 80, Tween 40 and chelator EDTA. The K_m and V_{max} values were found to be equal to 7.05 mM and 2.67 mmol/min, respectively. Thus, the lipase from *S. arlettae* may have considerable potential for industrial application from the perspectives of its tolerance towards industrial extreme conditions of pH, temperature, salt and solvent.

2.1 INTRODUCTION

Lipases (triacylglycerol acylhydrolases (E.C.3.1.1.3) are a class of hydrolases that catalyze the hydrolysis of triglycerides to glycerol and free fatty acids over an oil-water interface and can reverse the reaction in aqueous and non-aqueous media (Horchani et al., 2012). Microbial lipases also have been immensely used for biotechnological applications in dairy, detergents, and textile industries, as well as surfactant and oil-processing industries with application versatility from esterified products to pharmaceutical products (Tan et al., 2010). Among different microbial sources, bacterial lipases received much attention for their substrate specificity and their ability to function in extreme environments. Bacterial lipases are mostly inducible enzymes with requirement of triglycerides as inducers (Neihaya et al., 2012). These are mostly extracellular in nature and are produced mainly through submerged fermentation.

Submerged fermentation (SmF) holds tremendous potential for the production of lipases due to usage of this crude one directly as a lipase source for industrial application (Kirk et al., 2002 and Barbosa et al., 2011). Lipase production through SmF is dependent upon a number of factors including carbon and nitrogen sources, pH, temperature, aeration and inoculum size. Optimization of fermentation conditions for microbial lipase is of great interest since culture conditions influence the properties of the enzyme produced as well as the ratio of extracellular and intracellular lipases. Therefore, optimization of the lipase production has been focused on improving fermentation conditions such as carbon or nitrogen source, temperature, pH, aeration, using inducers and source of inoculum, etc. (Gupta et al., 2004 and Sharma et al., 2001). The major requirement for commercial lipases is thermal stability for resisting the chemical modifications caused by high temperatures employed in various industrial lipase catalyzed reactions due to the high melting points of the substrates (lipids). Stability in organic solvents is required since low-water systems based on organic solvents are necessary in order to provide conditions that favour the synthetic reaction over the normal hydrolytic reaction (Ahmed et al., 2009). This has drawn the interest towards thermophiles in both research and industries. Searching for new sources of lipases is justified by realizing variety of future applications requiring not only enzyme–substrate specificity but also process stabilities such as wide pH tolerance and high thermal stability. The stability of these enzymes in organic solvents has pushed them into the frontier areas of organic synthesis leading to the designing of novel drugs, surfactants, bioactive compounds. Industrial demand for the thermostable enzymes continues to stimulate the search of novel thermophilic microorganisms from different unexploited regions of the earth, as small numbers of bacterial strains producing thermophilic lipases have been reported in the last decade (Karatay and Donmez, 2011). Recently, a screening of lipase activity was carried out on halophilic bacteria from salt lake of Yuncheng, China, in this work reported a moderately halophilic strain LY7-8 (Li and Yu, 2012). Recent studies also reported lipolytic enzyme-producing thermophilic microorganism named *Bacillus thermoamylovorans,* isolated from a hot spring in Galicia (North Western Spain) (Deive et al., 2012).

In the present study, lipase production from *S. arlettae* through SmF has been carried out and partial purification has been made to characterize its stability in the presence of pH, temperature, salt conditions, organic solvents, surfactants, inhibitors and metal ions.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

p-nitrophenyl palmitate (Sigma –Aldrich,USA) , LB Broth, Miller (Merck, India) were used for the present study. EDTA, o-phenanthroline, PMSF, guanidine thiocyanate, β-6-mercaptoethanol was either of HPLC grade or AR grade was obtained from Merck.

2.2.2 Microorganism and culture maintenance

The bacterial strain, *Staphylococcus arlettae* JPBW-1 used in this study was isolated from the rock salt mine Darang HP, India and identified by 16S rRNA analysis. It was cultured on Luria agar and maintained at 37 ?C. The strain was subcultured every two weeks to maintain its viability.

2.2.3 Lipase production through submerged fermentation

Submerged fermentation was carried out by seeding the inoculum size (2 – 12 %) in Erlenmeyer flasks (250 mL) containing 100 mL of the L.B medium (10g of a casein enzymic hydrolysate, 5 g of yeast extract, 10 g sodium chloride per litre) (pH 7 - 12), supplemented with soybean oil (2 – 12 % v/v). Our preliminary results in the selection of 100 ml media has been tabulated under Table 2.1.

Table 2.1: Selection of amount of media in 250 ml conical flask for SmF. (Experimental conditions: Temperature-30 ^{o}C, Inoculum size- 10 %, Incubation period - 3 h, Agitation speed-100 rpm)

Vol. of media (ml)	Lipase activity (U/ml)
25	0.026 $\pm$ 0.60
50	0.05 $\pm$ 0.77
75	0.1 $\pm$ 0.58
100	0.19 $\pm$ 0.46
125	0.08 $\pm$ 0.84
150	0.035 $\pm$ 0.62

The effects of different carbon sources (coconut oil, olive oil, and soybean oil) were estimated in relation to enzyme activity. Lipases are inducible enzymes, generally produced in presence of lipids. In the present study soybean oil (lipid source) acts as an inducer for lipase production.

2.2.4 Lipase assay and protein estimation

Lipase activity was assayed quantitatively using *p*-nitrophenyl palmitate as the substrate according to the method described by kordel et al., 1991. One enzyme unit was defined as the amount of enzyme that liberates 1 µmol of 4-nitrophenol per minute under the assay conditions. Protein content of cell-free supernatant was determined according to modified Lowry method (Lowry et al., 1951) using bovine serum albumin as standard.

2.2.5 Partial purification of lipase

To precipitate lipase by ammonium sulphate experiment was conducted at 10 %, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 % and 90 % saturation of ammonium sulphate salt under stirring conditions and maintained for 60 min at 4 °C. The precipitate was collected by centrifugation at 6314 g for15 min and was dissolved in a minimum quantity of 50 mM Tris-HCl buffer (pH 8) and precipitated protein was dialyzed against buffer for 24 hr. Then the lipase assay and protein estimation was performed.

2.2.6 Characterization of lipase

The pH stability of lipase was tested by incubating the enzyme at different pH ranging from 4 to 12 using different buffers (0.5 M; acetate, phosphate, Tris-HCl and glycine and NaOH,)) at 35 ^{o}C for 1 h , following by standard enzyme assay and reported in terms of relative activity. The effect of temperature (30 – 90 °C) on alkaline lipase activity was determined by incubating the reaction mixture for 1 h and relative activity was calculated. To study the effect of NaCl concentration, aliquots of the enzyme were incubated with different concentrations of NaCl (0 – 25 %) for 60 min at 35 °C and relative activity was measured under standard assay conditions. The effect of organic solvents on lipase was determined by incubating enzyme solution in different organic solvents at 35 °C, for 1 hr. Effect of different surfactants on the lipase activity was investigated by pre-incubating the enzyme for 60 min at 35 °C in Tris-HCl buffer (50 mM, pH 8.0) containing 1 mM Tween 40, Tween 20 and Triton-X 100. To determine

the effect of different chloride salts of the metal ions (1 mM; Co^{2+}, Ca^{2+}, K^{+}, Mg^{2+}, Hg^{2+}, Fe^{2+}, Mn^{2+} and Na^{+}) on lipase activity was investigated by pre-incubating the enzyme with metal ion (1:1 ratio) for 1 h at 35 °C and then, relativity activity was determined. The effect of chelators (EDTA, o-phenanthroline) and denaturants (PMSF, guanidine thiocyanate, β-6-mercaptoethanol) on the lipase activity were examined at concentration of 1 mM at 1:1 ratio for 1 h at 35 °C, and relativity activity was determined. In all these cases, the control used was the untreated enzyme under the same experimental conditions and relative activity was calculated taking the value of control as 100 %. The Michaelis- Menten constant (K_m) and the maximum velocity for the reaction (V_{max}) were determined from Lineweaver-Burk plot.

2.3 RESULTS AND DISCUSSION

2.3.1 Selection of parameters for lipase production through SmF

2.3.1.1 Selection of incubation temperature

For selection of optimum temperature for the production of lipase, the temperature was varied from 25 °C to 50 °C, keeping the other process conditions same. It was seen that the maximal lipase activity (0.216 U/ml) has been seen at 35 °C (Fig.2.1).

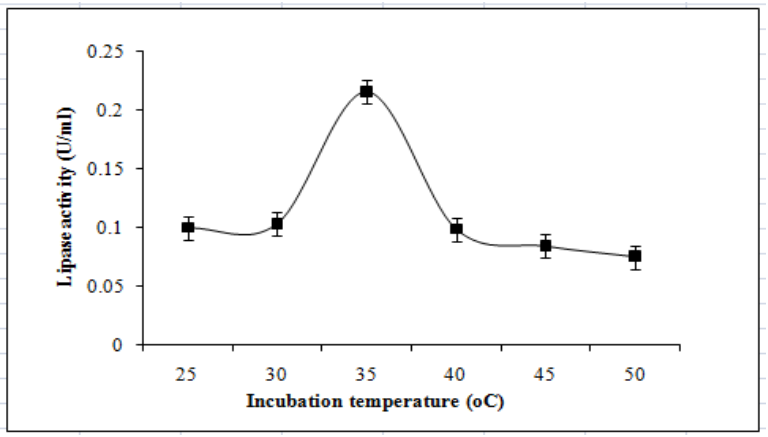


Figure 2.1: Selection of incubation temperature for lipase production. All values are represented as ± s.d of three replications. (Experimental conditions: Inoculum size- 5 %, Incubation period - 2 h, Agitation speed-100 rpm, pH- 7).

Mohan et al., 2008 found that an optimum temperature for lipase production by *Bacillus* sp. was 37 °C in his study that coincides with this study. Similarly, Walavalkar and

Bapal, 2002 have reported that, the lipase activity of *Staphylococcus* sp. was high at 37 °C. Thermophilic lipases have a high potential in the detergent and food industries and therefore, the organism may be exploited and scaling up could be attempted for industrial production.

2.3.1.2 Selection of inoculum size

The effect of inoculum size on lipase activity was determined at different inoculum size ranging from 2 % to 14 % at 35 °C. A maximum lipase activity of 0.349 U/ml has been observed using 10 % of inoculum. The cultures were incubated for 48 h and the lipase production was studied. After a certain concentration, the lipase activity increased and then decreased (Fig. 2.2). This may be due to overpopulated culture and fixed amount of nutrient with which the organism starts liberating proteolytic enzyme, enhancing self consumption (Basheer et al., 2011).

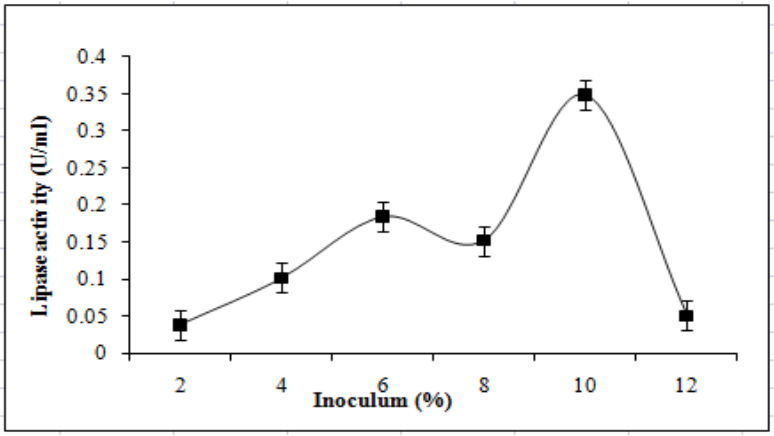


Figure 2.2: Selection of inoculum size for lipase production. All values are represented as ± s.d of three replications. (Experimental conditions: Incubation period- 2 h, Temperature - 35 °C, Agitation speed 100 rpm, pH - 7)

2.3.1.3 Selection of media pH

The pH of the production medium plays a critical role for the optimal physiological performances of the bacterial cell and the transport of various nutrient components across the cell membrane aiming at maximizing the enzyme yields. *Staphylococcus sp.* was inoculated in the lipase production medium and incubated at different pH (4-11). At pH 9, maximum lipase activity was observed (0.539 U/ml) (Fig.2.3). A comprehensive

review of all bacterial lipase done by Gupta et al., 2004 states that maximum activity of lipases at pH values higher than 7 has been seen in many cases. Generally, bacterial lipases have neutral (Dharmsthiti and Luchai, 1999 and Lee et al., 1999) or alkaline pH optima (Salihu et al., 2011; Kanwar et al., 2002; Sunna at al., 2002) with the exception of *P. fluorescens* SIK W1 lipase, which has an acidic optimum at pH 4.8 (Andersson et al., 1979)

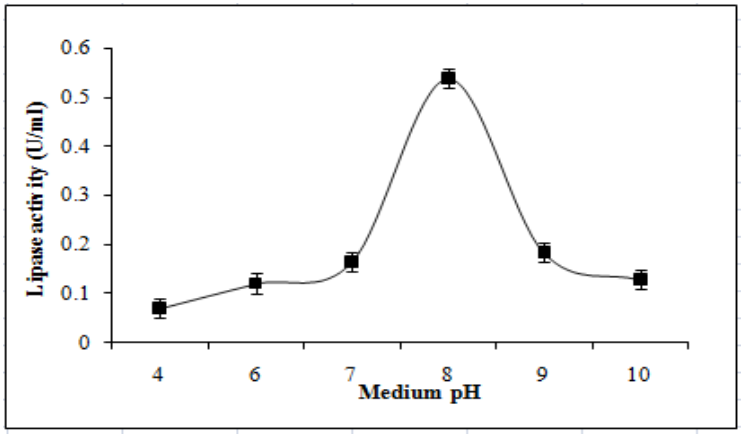


Figure 2.3: Selection of pH of media for lipase production. All values are represented as ± s.d of three replications. (Experimental conditions: Inoculum size – 10 %, Incubation period – 2 h, Temperature – 35 °C, Agitation speed- 100rpm)

2.3.1.4 Selection of incubation time

The effect of incubation time on lipase activity was determined for 1 – 15 hrs it was noted that high lipase activity (0.628 U/ml) has been found with incubation time of 3 h (Fig.2.4). At longer incubation periods, the lipase activity decreased which might be due to the depletion of nutrients, accumulation of toxic end products, and the change in pH of the medium, or loss of moisture. Several researchers have reported different incubation periods for optimal lipase production. Incubation periods ranging from few hours to several days have been found to be best suited for maximum lipase production by bacteria. The incubation period of 12 h was optimum for lipase production by *A. Bacillus sp.* RSJ1 (Sharma et al., 2002) and 16 h for *B. thermocatenulatus* (Schmidt et al., 1997)

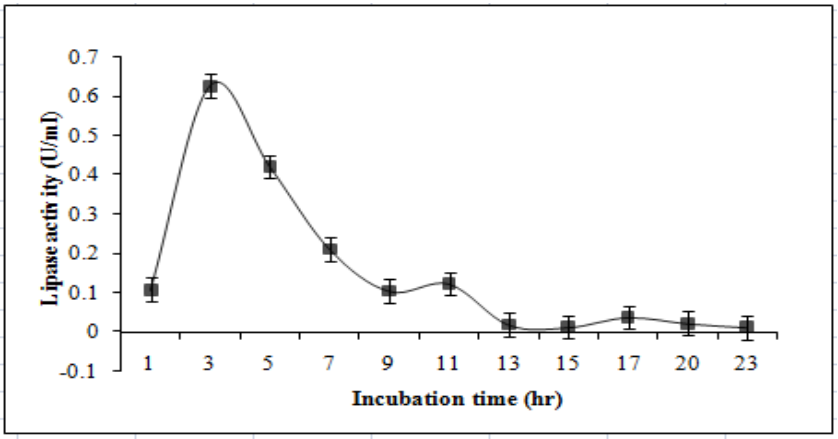
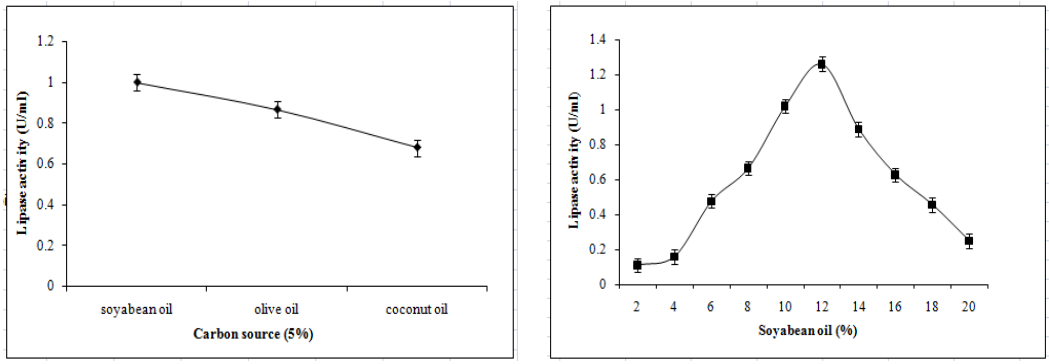


Figure 2.4: Selection of Incubation time for lipase production. All values are represented as ± s.d of three replications. (Experimental conditions: Inoculum size – 10 %, Temperature -35 °C, Agitation speed -100 rpm, pH-8.0)

2.3.1.5 Selection of carbon source

The culture environment has a dramatic influence on enzyme production especially carbon and nitrogen sources play a crucial role in enzyme induction, in bacteria. A major factor for the expression of lipase activity has always been carbon, since lipases are inducible enzymes and are thus produced in the presence of a lipid source such as oil or any other inducer, such as triacylglycerols, fatty acids, hydrolysable esters, Tweens, bile salts and glycerol.



(a) (b)

Figure 2.5: Selection of carbon source (a) and its % (b) for lipase production. All values are represented as ± s.d of three replications. (Experimental conditions: Inoculum size -10 %, Incubation period -3 h, Temperature – 35 °C, pH- 8.0, Agitation speed- 100rpm)

The increasing concentration of soybean oil increased the lipase activity (till 1.263 U/ml), but it has also been observed that, after a certain limit, the lipase activity fell down sharply (Fig 2.5). This may be attributed to the substrate inhibition (Rathi et al., 2001; Alfrod and Smith, 1965).

2.3.1.6 Selection of agitation speed

To evaluate the effect of agitation speed on lipase production by *S.arlettae*, experiments were carried out at different agitation speeds ranging from 50 to 200 rpm at 35 °C. It has been seen that, in the stationary condition, there was no lipase secretion, whereas, in shaking condition, a considerable amount of lipase activity was obtained when all other conditions were same. It was observed that maximum lipase activity has been observed (2.162 U/ml) utilizing an agitation speed of 100 rpm (Fig.2.6). From the results, the authors can interpret that the micro-organism responsible for lipase production had very strong affinity for oxygen for its metabolic activity particularly for the synthesis of lipolytic enzyme. This is not the first report which gave evidence of the negative effect of the higher mixing rates on the lipase production by *Geotrichum candidum.*

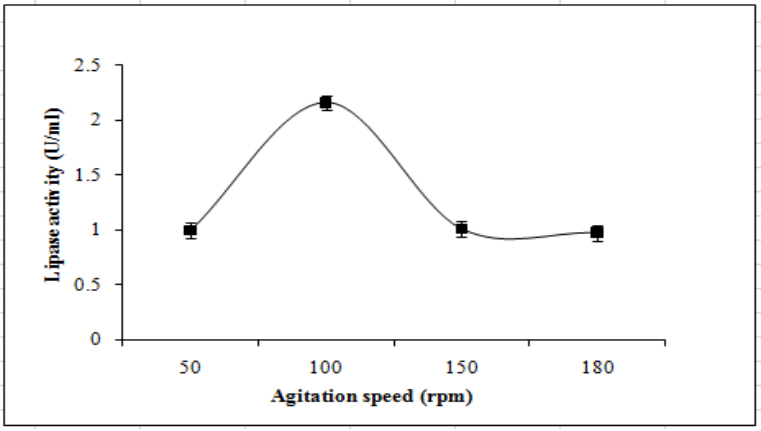


Figure 2.6: Selection of agitation speed for lipase production. All values are represented as ± s.d of three replications. (Experimental conditions: Inoculum size- 10 %, Incubation period -3 h, Temperature – 35 °C, pH-8.0, Carbon source- 12 % soybean oil)

Alford and Smith, 1965 reported that the lipase yields reduced for 60 % as a result of the mixing at low rates and even more at the higher ones. Wouters, 1987 similarly to the

previous case, reported that the growth and the lipase production by *Geotrichum candidum* decreased as the aeration or agitation rate of the culture medium increased.

2.31.7 Partial purification of lipase by ammonium sulphate precipitation

The ammonium sulphate precipitation was carried out by addition of varying concentration (10 % to 100 %) of ammonium sulphate under stirring conditions. The precipitated protein was collected through centrifugation (6987 g, 10 min) and dissolved in 50 mM Tris-HCl (pH 8.0) and assayed for lipase activity. It was found that at 60 % ammonium sulphate concentration, the specific activity of lipase was more. A purification fold of 3.72 and enzyme yield of 31 % was obtained. Chahinian et al., 2000 and Hiol et al., 2000 were reported the effective recovery of extracellular lipases from *Penicillium cyclopium* and *Rhizopus oryzae* respectively through ammonium sulphate precipitation. Salting out through ammonium sulphate stabilizes the proteins against denaturation, proteolysis and bacterial contamination (Roe, 2001).

2.3.2 Characterization of lipase

2.3.2.1 Effect of pH on lipase activity

Changes in pH will affect the protein structure and the enzyme activities (Ohnishi et al., 1994). The effect of pH on lipase activity is shown in Fig. 7, where lipase showed activity in the pH range of 7.0-12.0 The activity was highest at pH 9 (0.5 M Tris buffer). However lipase retained 99 % and 102 %, of relative activity for 24 h at pH 8, and 9, respectively and very less activity has been observed at pH 4 and pH 5.0 respectively. So the enzyme was only active in alkaline pH range. Kumar et al., 2005 reported that the purified lipase from *B. coagulans* BTS-3 was stable within a pH range of 8.0–10.5 with optimum activity at pH 8.5. Stability of the enzyme at different pH values was determined by measuring the residual activity after incubating the enzyme in the pH range of 4–12 for 1 h at 4 ^{o}C. The enzyme exhibited maximum stability at pH 9 and comparable stability also observed at pH 12 (91 %). Fig 2.7 depicts that the enzyme showed stability in the pH range of 7 to 12.

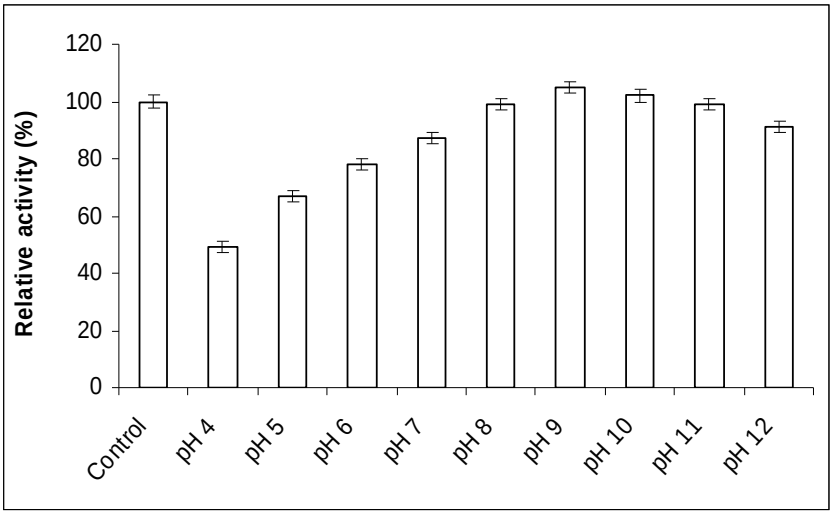


Figure 2.7: Effect of pH on *S. arlettae* lipase activity. All values are represented as ±SD of three replications

2.3.2.2. Effect of temperature on lipase activity

The effect of temperature on lipase activity was analyzed by carrying out assays at different temperatures (30 ^{o}C – 100 ^{o}C). With the rise in temperature, the enzyme activity started increasing and reached an optimum at 37 ^{o}C (Fig. 2.8). Our newly isolated *staphylococcus* sp. lipase was stable against thermal denaturation where it remained 62 % of its original activity at 90 °C after 60 min. Since thermostable lipases, which are active and stable in acidic and alkaline media, are very attractive and have a high potential for different industrial applications, this salt mine isolated *Staphylococcus* sp. lipase would be a potent and valuable enzyme for further applications. It has been reported that the drop in the percentage of residual activity at high temperatures results first in some conformational changes in the tertiary structure, and then almost complete inactivation of the enzymes (Ozen et al., 2004). In contrast to *Staphylococcus similans* lipase which is inactivated after a few minutes when incubated at 60°C (Sayari et al., 2001) *S.arlettae* retained 90 % or 60 % of its activity up to 60 min at 55 – 60 °C, respectively.

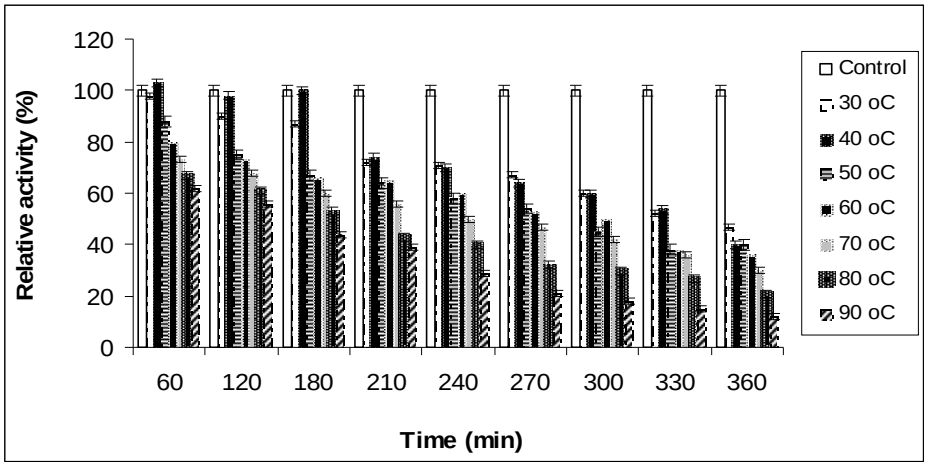


Figure 2.8: Effect of temperature on lipase activity. All values are represented as ± s.d of three replications

2.3.2.3 Effect of NaCl concentration on lipase activity

The effect of NaCl concentration lipase activity was studied by incubating the enzyme with different percentage of 0 to 30 % of NaCl. From Figure 2.9, it was observed that *S. arlettae* lipase could produce in 0- 25 % NaCl but the best growth was seen in medium without NaCl.

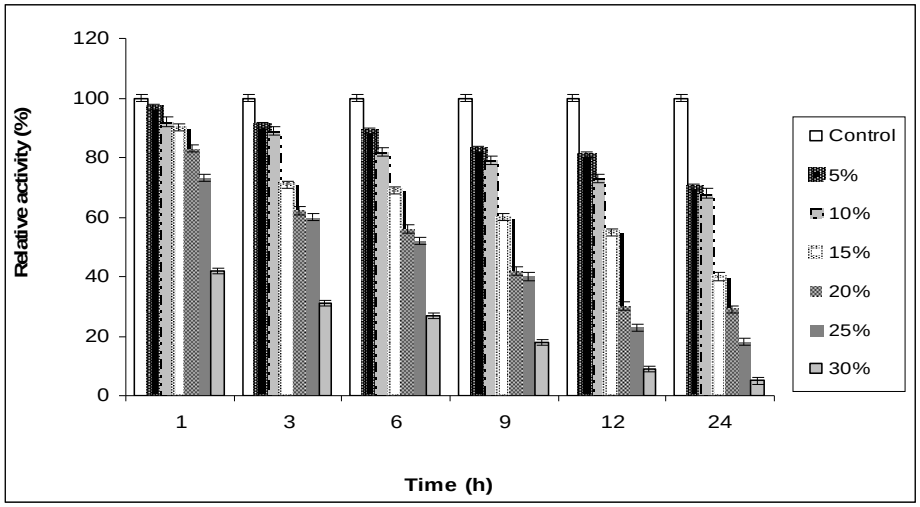


Figure 2.9: Effect of different NaCl concentration on lipase activity. All values are represented as ± s.d of three replications

This showed that *Staphylococcus arlettae* should be classified as halo-tolerant bacteria. Enzyme stability at high salt concentrations might indicate that the enzyme will be stable in the low water activity environments that occur in biocatalytic reactions carried out in organic solvents (Eichler, 2001).

2.3.2.4 Effect of organic solvents

Exposure of the *S.arlettae* to various organic solvents for 60 min showed that this enzyme retained activity in all organic solvent tested. The highest relative activity was achieved at 168.0 %, 143.0 %, and 141.8 % in xylene, benzene, and toulene, respectively (Fig 2.10). However, the activities were decreased when the enzyme incubation were extended to 2 h in organic solvents. Staphylococcus lipase was very stable when incubated with benzene, xylene, n-hexane, methanol, ethanol and toluene below 30 % (v/v) but was stability reduced drastically above 40 %. The stability of *S. arlettae* in aqueous-organic mixtures suggested the ability of this enzyme to retain activity in organic solvents and held the potential for its use in organic synthesis and related applications. High activity and stability of lipases in organic solvents is an essential prerequisite for applications in organic synthesis (Doukyu and Ogino, 2010; Ogino and Ishikawa, 2001). Hence stability in organic solvents are considered unique attributes in a lipase.

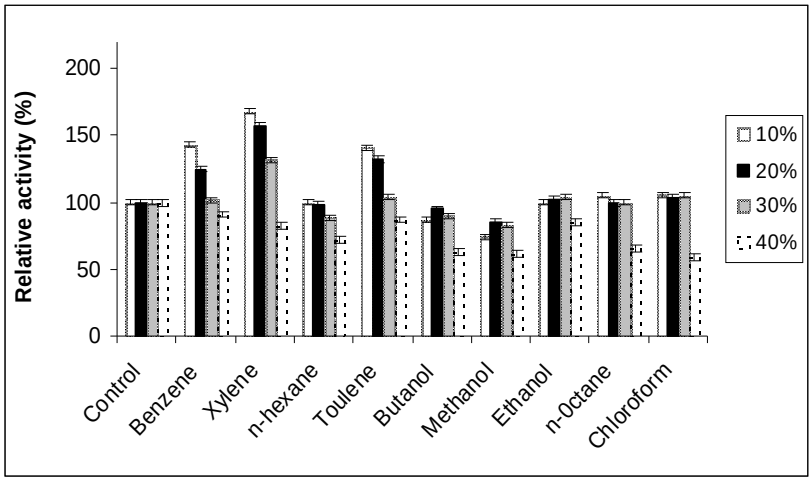


Figure 2.10: Effect of organic solvents on lipase activity. All values are represented as ± s.d of three replications

2.3.2.5 Effect of metal ions on lipase activity

The effect of different metal ions were tested on lipase activity at 1 mM concentration in 50 mM Tris HCl buffer (pH 8.0) for 1 hr. Previously, it has been demonstrated that the activity of *Staphylococcal* lipases may depend on the presence of Ca^{2+} ions (Sayari, 2001). It has been reported that the lipases from *P. glumae* (El et al., 2003) and *S. hyicus* (Rosenstein and Gotz, 2000) contain a Ca^{2+}-binding site which is formed by two conserved aspartic acid residues near the active-site, and that binding of the Ca^{2+} ion to this site dramatically enhanced the activities of these enzymes. Kambourova et al., 2003 suggested that the positive effect of Ca^{2+} is due to the formation of insoluble ion-salts of fatty acids during hydrolysis, thus avoiding the product inhibition.It was observed that, in the presence of 1 mM $CaCl_2$ and $MnCl_2$, the enzyme activity increased. All the other metal ions inhibited the activity of lipase (Fig. 2.11).

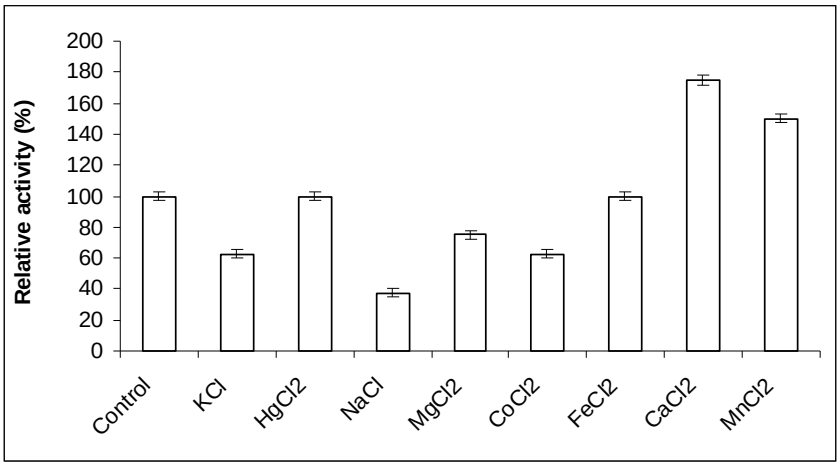


Figure 2.11 Effect of different metal ions on lipase activity. All values are represented as ± s.d of three replications

2.3.2.6 Effect of surfactants and vitamins on lipase activity

Surfactants usually increase the permeability of the cell wall. The higher concentration of surfactants may have adverse effects on the physiology of the organism causing lower yield due to partial denaturation of the enzyme. All the surfactants used in the present study had an inducing effect on lipase production. The effect was the maximum in presence of Tween 40 (Fig. 2.12). Stimulating effect of surfactants on enzymatic

hydrolysis has been reported many times (Kristensen et al., 2007). Ebrahimpour et al., 2011 and Castro-Ochoa et al., 2005 found that lipase activity of *Bacillus* sp. was enhanced in the presence of Triton X-100. The Lip-SBRN2 exhibited a high level of activity in the presence of SDS (Kanjanavas et al., 2010). It was observed that lipase production was affected in the presence of vitamins. Vitamins act as prosthetic groups for many enzymes. The lipase activity was found to be a maximum in the presence of nicotinic acid (Fig.2.13) Nicotinic acid stimulated maximum lipase in *Curvulariam pellescens, Fusarium equiseti* and *Trichoderma viridae* (Kakde and Chavan, 2011).

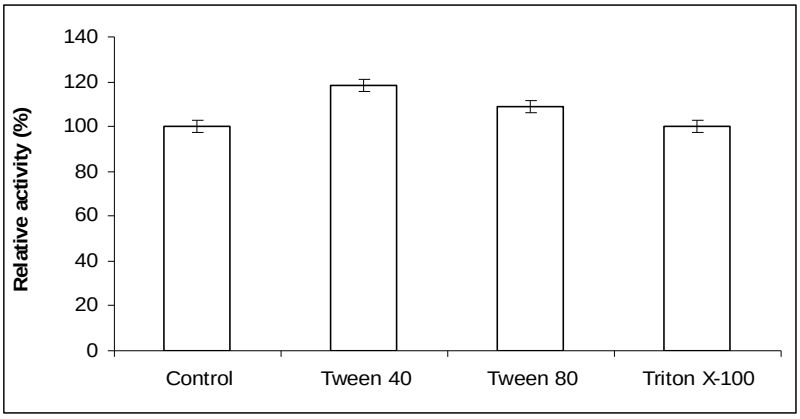


Figure 2.12 Effect of surfactants on lipase activity. All values are represented as ± s.d of three replications

2.3.2.7 Effect of inhibitors and chelators on lipase activity

The lipase of S. *arleatte* when incubated with 1 mM serine protease inhibitors, PMSF β-6-mercaptoethanol and guanidine thiocyanate for 1h showed drastic inhibition in lipase activity. Inhibition of activity with serine inhibitors shows that this lipase belongs to the class of serine hydrolases (Yadav et al., 1998 and Gilbert et al., 1991). The lack of sulphur containing amino acid in lipase active site has been confirmed through the lipase activity inhibition with β-mercapto ethanol and guanidine thiocyanate (Brzozowski et al., 1991). The chelators, EDTA and *o*-phenanthroline were studied for their influence on lipase activity at a concentration of 1 mM. The enzyme activity has been seen to increase in presence of 1 mM EDTA and lipase activity has been diminished with 1 mM *o*-phenanthroline (Fig. 2.14). Enhanced and diminished activities

in the presence of EDTA and *o*-phenanthroline has attributed to the non-requirement and requirement of cofactor for lipase activity respectively (Yadav et al., 1998 and Brzozowski et al., 1991).

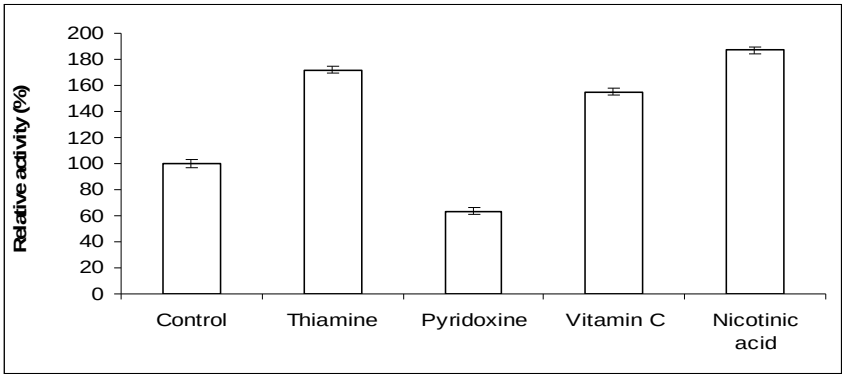


Figure 2.13 Effect of vitamins on lipase activity. All values are represented as ± s.d of three replications

The inhibition results with *o*-phenanthroline have been also reported in case of *Penicillium chrysogenum* (Bancerz et al., 2005).

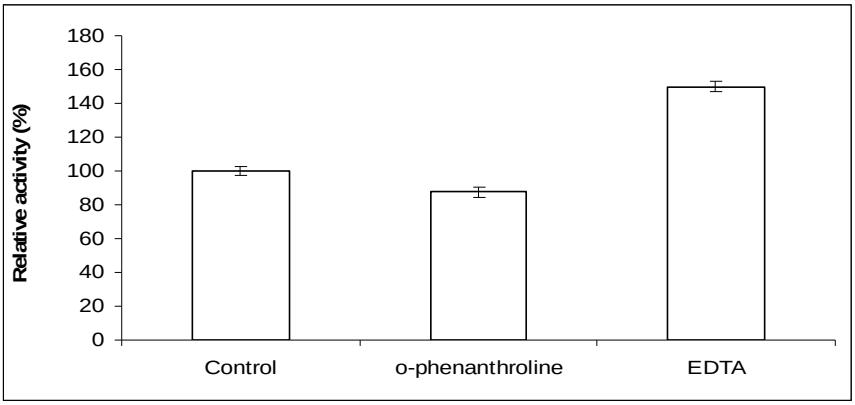


Figure 2.14 Effect of chelators on lipase activity. All values are represented as ± s.d of three replications

2.3.2.8 Kinetic constants of S. arleatte lipase

Basic enzyme kinetics such as K_m and V_{max} are adopted to describe the dynamic behavior. The kinetic constants K_m and V_{max} have been seen to be equal to 7.05 mM and 2.67 µmol/min, respectively for *S. arleatte* lipase using *p*-NPP as substrate at 35 °C, pH 8.0 through Lineweaver –Burk plot (Fig. 2.15).

The high affinity of the enzyme for *p*-NPP is reflected in the relatively low K_m value. Similar results have been reported in case of lipase from *Staphylococcus aureus* and *P. cepacia* using *p*-NPP as substrate. The kinetic constants of lipase from *Staphylococcus aureus* have been reported as 14.53 mM and 1485 µM/min/mg for K_m and V_{max} values, respectively (Horchani et al., 2009). Pencreac'h and Baratti (1996) reported K_m and V_{max} values of 12 mM and 30 mmol/min, respectively for *P. cepacia* lipase using *p*-NPP as a substrate.

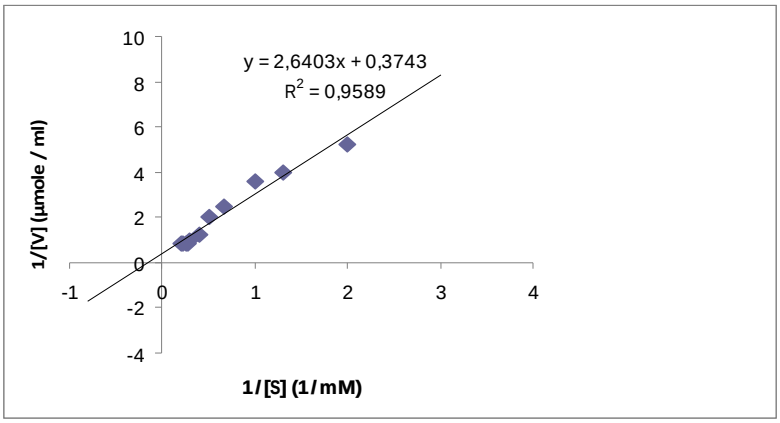


Figure 2.15: The Lineweaver-Burk plot of *S. arleatte* lipase

2.4 CONCLUSIONS

The properties of a novel, halo - thermo - solvent- detergent tolerant lipase by *Staphylococcus arlettae* showed many advantageous features for industrial applications, which may be helpful for possible application in the detergent industry, leather, pharmaceutical, cosmetic industry. This lipase was characteristically stable at 30–90 °C, pH 7.0–12 and 0 -20 % NaCl. Enzyme activity was stimulated by Ca^{2+}, Hg^{2+} and $Mn^{2+,}$ and inhibited by K^+, Zn^{2+}, and Co^{2+}. Additionally, the enzyme was strongly inhibited by PMSF, ß-mercaptoethanol but not affected by EDTA. The PMSF inhibition showed that the key enzyme was a serine hydrolase. All of these results led us to conclude that the enzyme may have considerable potential for industrial application from the perspectives of its properties.

CHAPTER 3

Modeling and optimization studies of lipase production through statistical integrated artificial intelligence approach

ABSTRACT

Microbial enzymes from extremophilic regions such as hot spring serve an important source for various stable and valuable industrial enzymes. The present paper encompasses the modeling and optimization approach for production of halophilic, solvent tolerant, alkaline lipase from *Staphylococcus arlettae* through response surface methodology integrated nature inspired genetic algorithm. Response surface model based on central composite design has been developed by considering the individual and interaction effects of fermentation conditions on lipase production through submerged fermentation. The validated input space of response surface model (with R^2 value of 96.6 %) has been utilized for optimization through genetic algorithm. An optimum lipase yield of 6.5 U/ml has been obtained using binary coded genetic algorithm predicted conditions of 9.39 % inoculum with the oil concentration of 10.285 % in 2.99 hrs using pH of 7.32 at 38.8 °C. This outcome could contribute to introduce this extremophilic lipase ((halo-, solvent-, tolerant) to industrial biotechnology sector and will be a probable choice for different food, detergent, chemical and pharmaceutical industries. The present work also demonstrated the feasibility of statistical design tools integration with computational tools for optimization of fermentation conditions for maximum lipase production.

3.1 INTRODUCTION

Hydrolases particularly lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) from extremophilic microorganisms are experiencing a growing demand, due to their versatile catalytic activities (regio- and entio- selectivity) coupled multifold industrial applications (Selvin et al., 2012). Among different sources, microbial lipases have gained unique industrial attention due to their stability, selectivity, broad substrate specificity and their cost effective production. The extracellular bacterial lipases are of considerable commercial importance, due to their substrate specificity, their ability to function in extreme environments and their bulk production is much easier. Currently,

bacterial lipases are in high demand because they tend to have alkaline pH optima and are often thermostable (Andualema and Gessesse, 2012; Jaeger, 1994).

Lipases from extremophiles are capable of functioning in the presence of salts, oxidizing agents, organic solvents and can withstand the harsh industrial conditions may permit their use in some specialized industrial applications, such as novel substrate catalysis reactions (Kapoor and Gupta, 2012). Production of lipases through submerged fermentation (SmF) avoids the unwanted metabolites production (usually produced under solid state fermentation) which facilitates easier downstream processing of lipases. Bacterial lipases are mostly extracellular and are greatly influenced by nutritional and physicochemical factors (Aires-Barros et al., 1994; Kim et al., 1996; Jaeger et al., 1999). The usual cumbersome one variable at a time approach (OVAT) of selecting fermentation conditions for enzyme production fails to give interaction effects of independent variables on the final production yield of enzymes. Response surface methodology (RSM) is a statistical coupled mathematical tool, in which the response of interest is influenced by several variables and the objective is to optimize this response and generates a mathematical model that describes the process by taking individual and interaction effects of the process variables (Myers et al., 2009). Several researchers acknowledged the modeling efficiency of RSM for different industrial enzyme production along with the bacterial lipases production from *Bacillus* and *Burkholderia* sp. (Kumar et al., 2011). Genetic algorithm (GA) is a powerful stochastic search and optimization technique work on "Survival of Fittest" concept of Darwinian Evolution, have received considerable attention and replacing the gradient based optimization approaches (Goldberg, 1989). It can be used to optimize fermentation conditions without the need of statistical designs and empirical models due to its flexibility in selection of objective function and constraints. The successful utilization of RSM integration with GA for enzyme production has been acknowledged in the case of lipase production from *Staphylococcus xylosus* (Khoramnia et al., 2010), and *Geobacillus sp.* strain ARM (Ebrahimpour et al., 2008). Having an extensive application of lipase in textile wet processing, food, chemical and pharmaceutical industries, and a continuous search for extremophiles that are capable of secreting lipolytic activities is underway.

Hence, in the present investigation we have utilized RSM integrated GA based approach for optimization of lipase production by extremophilic *S. arlettae* through submerged fermentation for the enhanced lipase yield.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

p-Nitrophenyl palmitate (Sigma-Aldrich, USA) , LB Broth, Miller (Merck, India) were used for the present study. All other solvents and reagents were either of HPLC grade or AR grade was obtained from Merck.

3.2.21 Microorganism and inoculum preparation

The strain *Staphylococcus arlettae* JPBW-1, previously isolated from rock salt mine Darang Mandi (Himachal Pradesh, India) and identified as a lipase producer, it was maintained on the slants of Luria agar and subculturing was done on every one week to maintain its viability. *Staphylococcus arlettae* JPBW-1 was cultivated in LB medium at 35 °C for 2 days for spore production.

3.2.3 Lipase production through submerged fermentation

Submerged fermentation was carried out by seeding the spore suspension (5ml) in Erlenmeyer flasks (250 mL) containing 50 mL of the LB medium, supplemented with soybean oil (12 % v/v). The flasks were incubated at 35 °C under agitation (100 rpm) for 3 h. After incubation, the fermentation medium was harvested by centrifugation at 6314 g for 10 min at 4 ^{o}C. The supernatant was collected and subjected to determine the lipase activity.

3.2.4 Lipase assay

The lipase activity was evaluated spectrophotometrically by measuring *p*-nitrophenol produced by hydrolysis of the *p*-nitrophenyl palmitate at 410 nm (Garlapati et al., 2010). One unit (U) of lipase activity was expressed as the amount of enzyme that liberates one micromole of *p*-nitrophenol released per minute under the assay conditions.

3.2.5 Modeling through RSM

RSM is a combination of mathematical and statistical techniques for empirical model building and optimization, which examines the relationships between one or more response parameters and a set of experimental input parameters.

This model is only an approximation, but it is used extensively because such a model is easy to estimate and apply, even when little is known about the process. RSM had not only been used for optimization of culture parameters in the fermentation process, but also for studying the combined effects of medium components (Khuri and Cornell, 1996).

3.2.5.1 Selection of process parameters for central composite design

Production of lipase through submerged fermentation mainly dependent on fermentation process variables namely temperature (30-40 ^{o}C), oil concentration (10-14), inoculum size (8-12 %), pH (7-9) and incubation time (2-4 h). In the present study based on the central composite design of RSM, design of experiments (DOE) was planned and performed for developing a polynomial response surface model after considering the above mentioned fermentation variables at three levels.

3.2.5.2 Statistical analysis

Non-linear regression analysis was carried out based on the data collected as per CCD (Table 3.1) planning for response, namely lipase activity using MINITAB 14 software which resulted in a second-order polynomial equation. The coefficient of the non-linear regression model can be determined using the method of least squares. The effect of the parameters and their interaction terms on the response has been studied by conducting the significance tests and Analysis of variance (ANOVA) has been carried out on each response to check the adequacy of the model. A detailed analysis of the effect of parameters and their interactions on the response was also made through the surface plots using MINITAB 14 software.

3.2.6 Artificial intelligence based binary coded GA optimization strategy

Optimization is described as the simulation performed aimed to maximize a particular process objective. The search for the desired optimum is usually done using

mathematical algorithms. In case of optimization, the problem of interest must be formulated as a mathematical model which describes the system and its performance. A simulation of the process with a mathematical model facilitates the process optimization against highly expensive experiments, predicting process results for any one set of decision variables. The simplicity, robustness, higher convergence rates in lesser computational time accounts for their popularity in solving the complex, non-linear problems. These algorithms differ with traditional and gradient based approaches, in searching a population of points in parallel not just a single point and utilization of the probabilistic transition rules instead of deterministic ones. In this context, the present work aims to optimize the non-linear RSM model of lipase production using artificial intelligence based GA approach. GA is the most popular EA (Evolutionary algorithm) which mimics the principle of natural evolution. In GA, the optimization search proceeds through three operators namely reproduction, crossover and mutation (Holland, 1992). The reproduction (selection) operator selects good strings in a population and forms mating pool. The chromosomes are copied based on their fitness value. No new strings are produced in this operation. Crossover operation generates a child chromosome by exchanging some portion of the strings (chosen randomly) with string of another chromosome in the mating pool using a crossover probability (Pc). If the child chromosome is less fit than the parent chromosome then it will slowly die in the subsequent generation. Mutation was the last operation of GA optimization and used further to perturb the child vector using mutation probability (Pm). It alters the string locally to create a better string and to create a point in the neighbourhood of the current point, thereby achieving a local search and maintaining the diversity in the population. The entire process is repeated till some termination criterion is met (Goldberg, 1989). The mechanics of GA is simply involving coping of the strings. This new population is further evaluated and tested for some termination criteria. In the present study, an attempt has been made to maximize the lipase activity of *S. arlettae* JPBW-1 using binary coded GA by utilizing the input space of the developed RSM model of lipase production through submerged fermentation. Taking ten bits for one variable, 50 bits (five input variables) were used to represent a GA string. Based on the concept of

duality, the maximization problem is converted to minimization problem. This simulation has been executed through C program.

3.3 RESULTS AND DISCUSSION

3.3.1 Modeling studies through RSM and statistical analysis

Temperature, pH, inoculum size, oil concentration and incubation time are the critical factors in SmF and their importance in enzyme production has been well established. One-factor-at-a-time approach was used to identify the concentration levels of these parameters. Most lipases are inducible enzymes and addition of oils proved to enhance lipase activity (Dandavate et al., 2009). RSM is a successive exploratory approach which allows the establishment of the relationship between multiple variables with obtained responses more efficiently than traditional design (Sifour et al., 2010). The process variables of SmF i.e., incubation temperature, pH, incubation time, inducer concentration (soybean oil %) and inoculum size (%) have been selected as input variables and experiments have been executed based on CCD for developing a second order polynomial response surface model for lipase production by *S.arleatte* (Table 3. 1).

These experiments were performed in triplicates and lipase activity (La) of *S.arlettae* JPBW-1 has been expressed as a non-linear function of the input process parameters in coded form as follows:

$$La = 3.52179 - 0.01167X_1 + 0.11833X_2 - 0.10222X_3 + 0.51389X_4 + 0.01222X_5 - 0.18187X_1X_2 - 0.28062X_1X_3 + 0.04188X_1X_4 + 0.04188X_1X_5 + 0.32312X_2X_3 + 0.08312X_2X_4 + 0.10313X_2X_5 - 0.01062X_3X_4 - 0.03562X_3X_5 - 0.15813X_4X_5 - 0.07564X_1^2 + 1.57436X_2^2 - 0.70064X_3^2 + 0.48436\ X_4^2 - 0.56064\ X_5^2 \quad \text{--------} \quad \textbf{(3.1)}$$

Where X_1, X_2, X_3, X_4 and X_5 represents temperature, oil concentration, inoculum size, pH and incubation time respectively.

Table 3.1: Central composite design with the experimental, predicted responses, and its R-studentized residuals.

Run Order	Input parameters					Response		
	Temper-ature(^oC) (X_1)	Oil concent-ration (X_2)	Inoculum Size (X_3)	Ph (X_4)	Incubati-onTime (X_5)	L.A (U/ml) (Experi-mental)	L.A (U/ml) (predicted)	R-studentized residual
1	35	12	10	8	4	3.06	2.97338	0.47939
2	30	14	12	7	4	4.62	4.70847	-2.19524
3	30	10	12	9	4	3.86	3.86208	-0.04236
4	30	10	8	7	4	3.74	3.76124	-0.43717
5	30	14	12	9	2	5.68	5.7218	-0.88478
6	35	12	10	8	3	3.26	3.52179	-1.11168
7	35	10	10	8	3	4.92	4.97782	-0.31798
8	40	14	8	9	2	4.87	4.84291	0.5608
9	40	14	8	7	4	3.91	3.92958	-0.40229
10	35	12	10	8	3	3.27	3.52179	-1.0643
11	35	12	8	8	3	2.9	2.92338	-0.12802
12	40	10	8	9	4	5.38	5.31319	1.50899
13	30	12	10	8	3	3.71	3.45782	1.53354
14	40	10	12	9	2	4.33	4.2943	0.74778
15	35	12	10	8	3	3.26	3.52179	-1.11168
16	35	12	10	8	3	3.86	3.52179	1.49949
17	30	10	8	9	2	4.78	4.75458	0.52531
18	35	12	10	9	3	4.42	4.52004	-0.5558
19	30	14	8	7	2	3.16	3.22097	-1.35149
20	40	10	8	7	2	4.39	4.37347	0.33895
21	40	12	10	8	3	3.37	3.43449	-0.35508
22	30	10	12	7	2	3.39	3.44236	-1.13374
23	40	10	12	7	4	3.19	3.20097	-0.22411
24	35	14	10	8	3	5.46	5.21449	1.48392
25	40	14	12	9	4	4.7	4.70041	-0.00838
26	40	14	12	7	2	3.15	3.20069	-1.09325
27	35	12	12	8	3	2.93	2.71893	1.24059
28	30	14	8	9	4	4.56	4.57069	-0.2184
29	35	12	10	8	2	3.05	2.94893	0.56166
30	35	12	10	7	3	3.78	3.49227	1.81538
31	35	12	10	8	3	3.35	3.52179	-0.70512
32	35	12	10	8	3	3.38	3.52179	-0.57742

Based on the significance test results (Table 3.2), the p values of X_4, X_2^2, X_3^2, X_4^2, X_1X_2, X_1X_3, X_2X_3, X_4X_5 and X_5^2 (Found to be less than 0.05, considering 95 % (a = 0.05) as a level of confidence) are considered as significant terms with impact on final lipase activity.

Table 3. 2: Results of significance test on the non-linear model-coefficients, standard errors, T statistics, and P values for the lipase activity (coded form) .

SI. No.	Terms	Coeffecient	Standard Error Coeffecient	T	P
1	Constant	3.528	0.071	49.618	0.000
2	X_1	-0.0117	0.058	-0.199	0.846
3	X_2	0.118	0.058	2.021	0.068
4	X_3	-0.102	0.058	-1.746	0.109
5	X_4	0.514	0.058	8.778	0.000
6	X_5	0.012	0.058	0.209	0.838
7	X_1^2	-0.075	0.158	-0.478	0.642
8	X_2^2	1.574	0.158	9.944	0.000
9	X_3^2	-0.707	0.158	-4.425	0.001
10	X_4^2	0.484	0.158	3.059	0.011
11	X_5^2	-0.56	0.158	-3.541	0.005
12	X_1X_2	-0.182	0.062	-2.929	0.014
13	X_1X_3	-0.287	0.062	-4.52	0.001
14	X_1X_4	0.049	0.062	0.674	0.514
15	X_{1X5}	0.041	0.062	0.674	0.514
16	X_2X_3	0.323	0.062	5.204	0.000
17	X_2X_4	0.083	0.062	1.339	0.208
18	X_2X_5	0.103	0.062	1.661	0.125
19	X_3X_4	-0.01	0.062	-0.171	0.867
20	X_3X_5	-0.035	0.062	-0.574	0.578
21	X_4X_5	-0.158	0.062	-2.547	0.027
	SS= 0.2484	R-sq =96.6%	R-sq(adj)=90.5%		

The *P*-value of the factors X_1, X_2, X_5, and X_2X_5 is found to be more than the confidence level (0.05) but their square terms P value are found to be less than the confidence level indicating its non-linear relationship with the response, lipase activity. The significant contribution of linear, square and interaction terms towards the response, lipase activity has been revealed through ANOVA results (Table 3.3), where the P values of all the terms were found to be less than the significance level α 0.05. The coefficient of multiple regression (R^2) was seen to be equal to 96.6 % which shows the developed model is an adequate predictor of the experimental conditions and confirmed that the

selected SmF process variables significantly influence lipase yield (Myers et al., 2009). Moreover, R^2 was found to be in reasonable agreement with adjusted R^2 (90.5 %).

Table 3.3: Results of ANOVA- lipase activity

Source	DF	Sequential SS	Adjusted SS	Adjusted MS	F	P
Regression	20	19.5268	19.5268	0.97634	15.83	0.000
Linear	5	5.1987	5.1987	1.03975	16.86	0.000
Square	5	10.1093	10.1093	2.02185	32.78	0.000
Interaction	10	4.2188	4.2188	0.42188	6.84	0.002
Residual Error	11	0.6785	0.6785	0.06168		
Lack –of-Fit	6	0.4080	0.4080	0.06800	1.26	0.410
Pure Error	5	0.2705	0.2705	0.05411		
Total	31	20.2053				

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The non-linear nature of SmF production variables interaction with lipase activity (U/ml) has been further seems through the response surface plots, which were depicted in Fig 3.1. Higher lipase activities have been attained at near +1 level value of temperature with oil concentration (Fig.3.1a) and pH (Fig. 3.1c). The cumulative effect of temperature with inoculums size and inoculation time on lipase activity has been depicted in Fig. 3.1 (b) & (d), where lipase activity (U/ml) increases initially with time till the mid value (0) and then starts to decrease to the higher level (+1). A similar trend has been followed in case of Fig. 3.1 (e), (g) and (j) response surface plots, where the interaction effect of oil concentration with inoculums size & incubation time and pH with incubation time on lipase activity has been explained. In these cases, higher lipase activities have been observed around high range (+1) values. The interaction effect of oil concentration and pH with lipase activity has been depicted in Fig. 3.1 (f), where higher lipase activities has been observed after mid value (0) range. Higher lipase activity has been observed around higher level (+1) in case of inoculums size and pH interaction on lipase activity (Fig 3.1 (h)). In case of inoculum size and incubation time interaction on lipase activity (3.1 (i), higher lipase activity has been observed near their zero levels. The significant effect of SmF production variables interactions on lipase activity suggesting their profound role in enhancing lipase activity under SmF conditions.

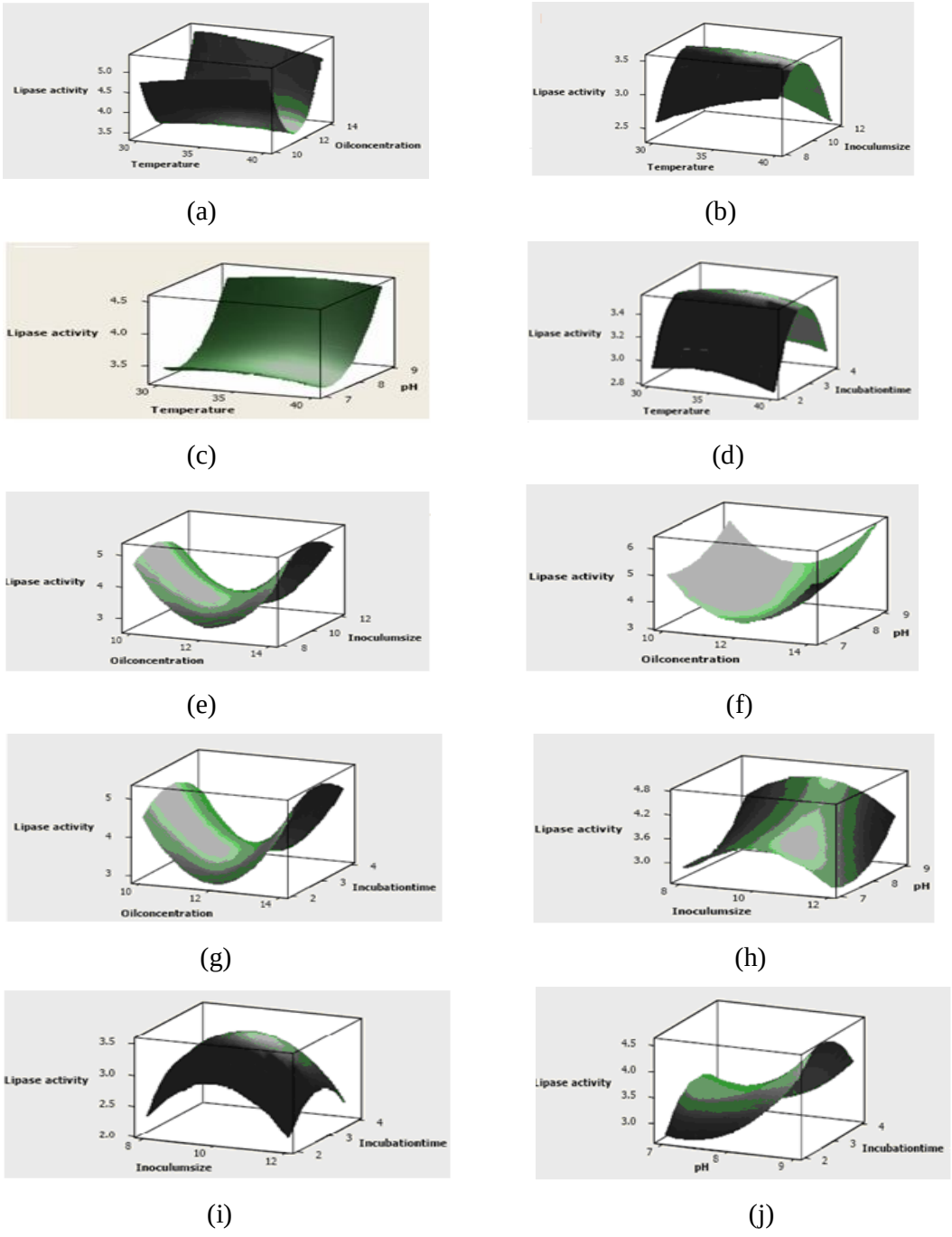

Figure 3.1: Surface plots of lipase activity (U/ml) with: (a) Temperature (°C) and oil concentration (%) (b) Temperature (°C) and inoculums size (%), (c) Temperature (°C) and pH, (d) Temperature (°C) and incubation time (h), (e) Oil concentration (%) and inoculums size (%) , (f) Oil concentration (%) and pH, (g) Oil concentration (%) and incubation time (h) (h) Inoculum size (%) and pH, (i) Inoculum size (%) and incubation time (h), (j) pH and incubation time (h).

The non linear relationship of fermentation process variables namely inoculum concentration and temperature on lipase yield from *Geobacillus thermoleovorans* CCR11 has been also found through the RSM approach (Sánchez-Otero et al., 2011). Significant and combined effects of polydimethylsiloxane (PDMS) and oxygen volumetric mass transfer coefficient through RSM has been acknowledged by Rech et al., 2011 for lipase production by *Staphylococcus warneri* EX17.

3.3.2 GA based optimization

GA is a search and optimization technique developed by Holland, 1992, which mimics the principle of natural evolution exploring the search space by incorporating a set of candidate solutions in parallel and solve complicated optimization problems by simulation (Sarkar and Modak, 2003). The application of genetic algorithms in bioprocess optimization had been reported by researchers which are more flexible tool had been used here for minimization of reaction time while maximizing product concentration (Zang et al., 2010). The selection of population size, number of generations, mutation probability and crossover mechanism play an important role in exploring the input space of the problem of interest by GA. In the present study, RSM model of lipase extraction is posed as an optimization problem for maximizing the lipase activity. A systematic study was conducted to determine the GA-parameters responsible for optimal value of lipase activity. The results of parametric study of GA have been shown in Fig. 3.2 for searching optimal fermentation process variables to predict the final lipase yield. The results of parametric study of GA have been shown in Fig.3.2 for searching optimal fermentation process variables to predict the final lipase yield. The Fig.3.2 shows the parametric analysis of Pm (0.001-0.0031), Population size (10-350) and Maximum Generations (25-1000) Vs Fitness value, from this analysis we have selected the optimum values of Pm, population size and maximum generation number at which optimum fitness value has been noticed one by one.

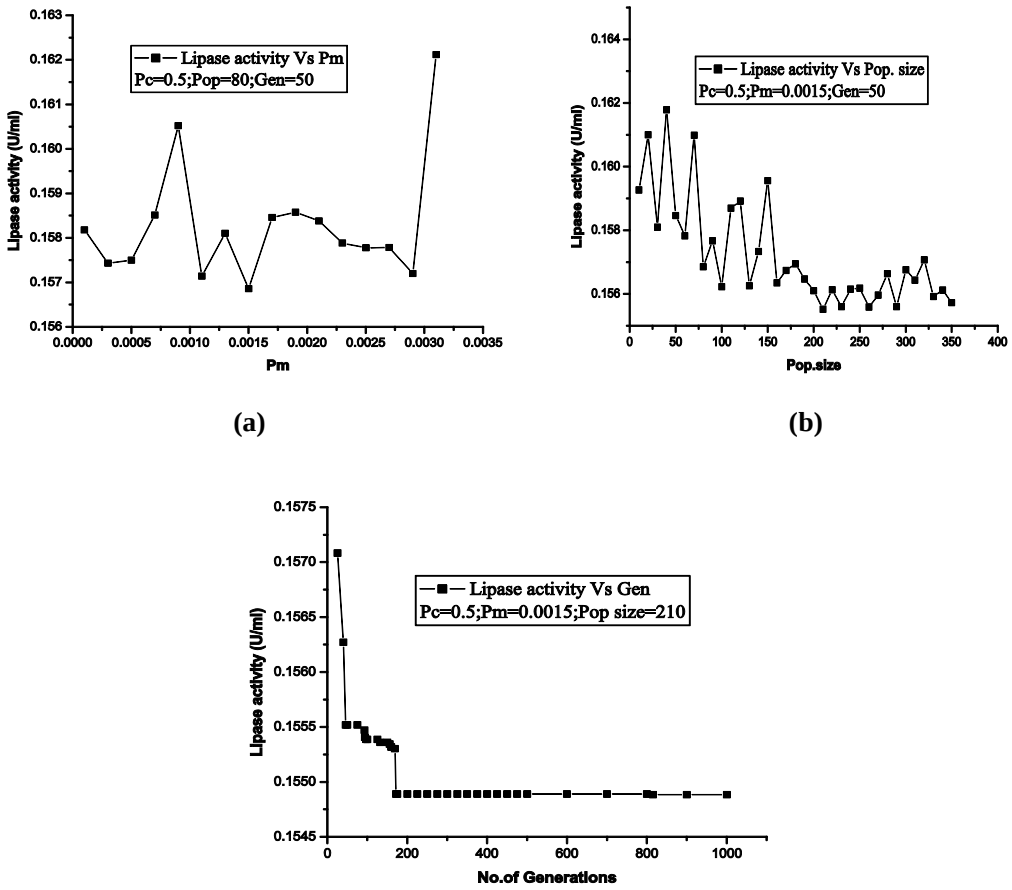

Figure 3.2: Results of parametric study of GA (a) Lipase activity Vs Mutation probability (Pm) (b) Lipase activity Vs Population size (c) Lipase activity Vs Maximum number of generations .

A tournament size of two and uniform crossover probability of 0.5 has been utilized throughout the binary coded GA search variables namely mutational probability, population size and No.of generations respectively. Once selected the bit-wise mutational probability (P_m) i.e., 0.0015, this has been utilized for further search in GA variable, population size. Finally, parametric study for No. of generations has been executed in the range of 25-1000. A maximum lipase production value of 6.456375 U/m has been attained using the GA parameters of 0.0015 Pm, pop size of 210 in 815 generations. Decoding of 50 bit length string (10 bits to each SmF variable, 10 x 5, obtained through the final GA run using the optimal GA parameters) to real SmF variable has been done through linear mapping. The predicted optimum SmF variables

were seen to be equal to 38.82 °C, 10.285 %, 9.392 %, 7.32, 2.995 h for temperature, oil concentration, inoculums size, pH and incubation time respectively.

3.3.2.1 Experimental validation of GA proposed optimization results

The GA optimized parameters obtained have been validated experimentally by conducting the experiment in triplicates. The extracellualr lipase production under the GA stated conditions resulted in the lipase activity of 6.50U/ml, which was found to be in close agreement with the GA predicted value 6.54U/ml. The lipase yield improved about 1.8 fold than the one at a time approach for optimization of lipase production in which the lipase yield was 3.54U/ml. The significant correlation between predicted and observed values of the lipase yield in these experiments justified the accuracy of the binary coded GA prediction. Among the various artificial intelligence techniques, genetic algorithms, a powerful stochastic search and optimization technique, have received considerable attention. Genetic algorithms can be used to optimize fermentation conditions without the need of statistical designs and empirical model. Implementation of a GA for multiobjective experimental optimization was recently demonstrated (Link and Weuster-Botz, 2006) and, thus, offers a chance for further reduction of the experimental effort. Ebrahimpour et al., 2008 have also utilized artificial intelligence techniques for enhanced lipase production from a newly isolated thermophilic *Geobacillus sp*. strain ARM. The better search criteria of GA´s for optimal conditions have been acknowledged in the case of polyhydroxybutyrate (PHB) production by *Azohydromonas lata* MTCC 2311 (Zafar et al., 2012) and in laccase mediated biodegradation of 2,4 – dichlorophenol (Bhattacharya et al., 2009). Moreover, preliminary results showed that lipase from *Staphylococcus arlettae* JPBW-1 showed stability in the presence of salt (up to 30 % NaCl), organic solvents (up to 30 % benzene, xylene, n-hexane and toluene) and has ability to work in extreme conditions of temperature (up to 70 ^{o}C) and pH (8-12) (Chauhan and Garlapati, 2013).

3.4 CONCLUSIONS

To summarize, this study presents the evaluation of RSM integrated GA in modeling and optimization of lipolytic activity production from *S. arlettae.* A second order polynomial response surface model has been developed successfully and utilized in search of optimal conditions for lipase production through SmF using binary coded GA. The optimum fermentation conditions obtained for the synthesis of lipase from *S.arlettae* were 38.8 ℃, oil concentration 10.2 %, inoculum volume 9.3 %, pH 7.32 and incubation time 3 h for obtaining a maximum lipase activity of 6.45 U/ml. An overall 1.8-fold increase in lipase activity was achieved after fermentation variables optimization, following the statistical approach. The high tolerance of this lipolytic enzyme under extreme conditions will be an enzyme of choice for many industries and considered to be a good candidate of its viability for commercialization.

CHAPTER 4

Evaluation of lipase for its detergent additive capability

ABSTRACT

Lipases are the enzyme of choice for many laundry detergent industries owing to their ability to remove triglycerides from the soiled fabric which reduces the usage of phosphate based chemical cleansers in the detergent formulation. A partially purified bacterial lipase from *Staphylococcus arlettae* JPBW-1 isolated from the rock salt mine has been assessed for the removal of triglyceride soil by developing a presoak formulation so as to use the enzyme as an additive in laundry detergent formulations. The effects of selected surfactants, commercial detergents and oxidizing agents on lipase stability were studied in a preliminary evaluation for use in detergent formulation. Partially purified lipase has been shown good stability in presence of surfactants, commercial detergents and oxidizing agents. Washing efficiency has been found to be enhanced while using lipase with 0.5 % non ionic detergent than the anionic detergent. The wash performance using 0.5 % Wheel with 40 U lipase at 40 °C within 45 min results in maximum oil removal (62 %) from the soiled cotton fabric. Hence, the present study opens the new era in enzyme based detergent sector for formulation of chemical free detergent using the alkaline bacterial lipase.

4.1 INTRODUCTION

Lipases (triacylglycerol acyl hydrolase, E.C. 3.1.1.3.) are ubiquitous enzymes with industrial potential of synthesizing structural triglycerides, which are used as detergents and emulsifiers in nutrition and cosmetics (Andualema and Gessesse, 2012). Detergent enzymes constitute about 32 % of the total worldwide industrial enzyme production (Lomax et al., 1997). A major requirement for commercial lipases is thermal stability which would allow enzymatic reaction to be performed at higher temperature and would be helpful to increase conversion rates and substrate solubility. The importance of alkaline and thermostable lipases for different applications has been growing rapidly (Cherif et al., 2011). The usage of alkaline lipases in detergent formulations enhancing substantially which facilitates alternative for the phosphate builders in chemical detergent which are considered as major pollutants from detergent industry. Ideally, alkaline lipases in a detergent formulation should be stable over a broad range of

temperature, pH and compatible with surfactants, oxidizing agents at lower concentrations with broad substrate specificity (Jellouli et al., 2011).

The detergent industries are relying on recombinant lipases (Lipex® and Lipolase ®from Novozymes) for formulation of bio-detergents due to the suitability of these lipases for harsh conditions of detergent formulation ingredients such as surfactants, oxidizing agents (Saeki et al., 2007). Researchers are in search of enzymes from indigenous extremophilc regions for better application in laundry detergent industry. Among different sources (fungal, yeast, bacterial), bacterial lipases received much attention for their substrate specificity and their ability to function in extreme environments of temperature, pH and surfactant and oxidizing agents tolerance (Horchani et al., 2012). Rathi et al., 2001 showed the application of bacterial lipase *Burkholderia cepacia* as an additive in detergent formulation which exhibits better stability towards commercial detergents and oxidizing agents in comparison to commercial Lipolase. In another study, Thirunavukarasu et al., 2008 have shown the use of *Cryptococcus sp.* S-2 lipase in detergent formulation and optimized washing conditions through response surface methodology. Moreover, bacterial lipases added to household detergents reduce or replaces synthetic detergents, which have considerable environmental problems (Basketter et al., 2012). Ideally, alkaline lipases are suitable candidates as a detergent additive for formulating a presoak formulation in detergent industry (Hasan et al., 2006). Hence, in the present study we are introducing an alkaline bacterial lipase produced by *Staphylococcus arlettae* JPBW-1. Lipase was further investigated in order to assess their compatibility with several other commercial surfactants, oxidizing agents and the well known commercial detergents and tested for its washing efficiency for removal of olive oil from soiled cotton fabric.

4.2 MATERIALS AND METHODS

4.2.1 Microorganism, chemicals and reagents

Staphylococcus arlettae JPBW-1 was used for the lipase production, which was isolated from the one and only rock salt mine of India, Darang, HP and deposited in MTCC, Chandigarh as *Staphylococcus arlettae* JPBW-1 MTCC5589, maintained on Luria agar

slants at 4 ^{o}C. Olive oil used was the brand of Sos Cuetara, S.A. Figaro. Surfactants used were selected from the commercially available products; as nonionic surfactants: Tween 80 and commercial detergents of Indian market namely Ariel, Tide, Wheel active and Nirma (Procter and Gamble Home Products Ltd.), Rin Magic and Surf Excel (Hindustan Lever Ltd.), Sodium dodecyl sulphate (SDS) as anionic surfactant. *p*-nitrophenyl palmitate (*p*-NPP) was procured from Sigma, USA.

4.2.2. Lipase production and partial purification

Bacterial lipase was produced through submerged fermentation by cultivating 100 ml inoculum (48 h old) supplemented with 8 % soybean oil in a shaking flask (250 ml) with 100 ml of the LB broth medium. The culture was incubated for 3 h on a rotary shaker (125 rpm) at 37 °C. After 3 h, the fermented broth was centrifuged at 5367 g for 15 min at 4 °C and the cell-free supernatant was used for estimation of lipase activity and partial purification (Ammonium sulphate precipitation, 60 %). The lipase has pH optima of 11.0 and activity in a broad temperature range of 25- 90 °C (Chauhan and Garlapati, 2013).

4.2.3 Lipase assay

Lipase activity was determined using *p*-NPP as substrate (Garlapati and Banerjee, 2010). One unit (U) of lipase activity was expressed as the amount of enzyme that liberates one micromole of *p*-nitrophenol released per minute under the assay conditions.

4.2.4 Compatibility of lipase with surfactants and commercial detergents

To investigate the compatibility of lipase in various surfactants and commercial detergents, respective surfactants and detergents were added to the reaction mixture at a concentration of 7 mg/ml and assayed under standard assay conditions and expressed as percent relative activity. The endogenous lipases contained in these detergents were inactivated by heating the diluted detergents for 1 h at 65 ^{o}C prior to the addition of the enzyme preparation. To determine the stability, an aliquot of enzyme sample (50 U/ml) was incubated with equal volume of detergent solution (7 mg/ml of respective detergent) in Tris- HCl buffer (0.1 M, pH 8.0) for 1 h at 30 ^{o}C. The relative activity (%)

of each sample was determined and compared with the control without detergent. The relative activity of control was defined as the enzyme activity without detergent, incubated under the similar conditions and was taken as 100 %.

4.2.5 Compatibility of lipase with oxidizing agents

Lipase (50 U/ml) compatibility in presence of oxidizing agents was determined in Tris-HCl buffer (0.1M, pH 8.0) containing 0.5–2.0 % (v/v or w/v) of hydrogen peroxide, sodium perborate and sodium hypochlorite for 1 h at 30 °C and relative activity was estimated and compared with the control without oxidizing agent. The relative activity of control was defined as the enzyme activity without oxidizing agent, incubated under the similar conditions and was taken as 100 %.

4.2.6 Preparation of olive oil soiled cotton fabric

The cotton fabric (5cm X 10cm) to be soiled was highly defatted in boiling chloroform for four hr. This treatment was repeated three times. The cotton fabric was soiled by spotting with 0.5 ml of olive oil benzene solution (100 mg/ml conc.) with micropipette two times.

4.2.7 Preparation of washing solution

Four kinds of washing solution were prepared their compositions are shown in Table 4.1 for making 100 ml of respective washing solution. Solution B-D-L, which contained the buffer solution, the surfactants solution and the lipase solution, was prepared in the following manner. The buffer solution and the surfactant solution were measured into an Erlenmeyer flask with ground stopper and preheated at 37 °C for 10 min, followed by the addition of the lipase solution. Solutions B-L, B-D and B were prepared in the same manner. The volume of the final solutions was adjusted to 100 ml by adding distilled water. Then, 10 pieces of the soiled fabric was put into flask. For selecting the best process condition (conc. of detergent, activity of lipase, washing temperature and washing time) initially washing compositions of Table 4.1 was used. After selection of one best process condition through changing one variable at a time approach, the best condition was used for the selection of next process condition by utilizing the proportion of Table 4.1 for making the detergent solutions.

Table 4.1: Washing solutions and its composition for making 100 ml of washing solution

Components	Volume(ml)			
	B^a	B^a+ L^b	B^a+D^c	B^a+ D^c+ L^b
Tris HCL (0.1M, pH8.0)	40	40	40	40
Detergent (0.5%)	-	-	50	50
Lipase (50U/ml)	-	10	-	10
Distilled water	60	50	10	-

[a] Buffer; [b] Lipase ; [c] Detergent

4.2.8 Washing procedure and olive oil determination

The soiled fabric was washed at 37 °C for 20 min by incubator with the shaking of 100 rpm. The fabric was rinsed thrice each rinsing was with 100 ml of distilled water at 37 °C for 2 min. Then, fabric was air dried. Washing also was done at different temperature and time interval, as well as by using the lipase solution and the detergent solution, both in different concentrations. Olive oil was extracted from the fabric with petroleum ether for six hours in Soxhlet extractor. After petroleum ether was completely evaporated from the extract and weight of olive oil was determined.

Calculation of removal: The removal of olive oil was calculated by equation, based on the weight of the total fatty acids on the fabric before and after washing.

$$\text{Removal (\%)} = (W_i - W_r) \times 100 / W_r$$

Where W_i = weight of total olive oil before washing (mg) and W_r= weight of total olive oil after washing (mg).

4.3 RESULTS AND DISCUSSION

4.3.1 Compatibility of lipase with surfactants and commercial detergents

For effective use under harsh detergent industry conditions, lipolytic enzyme must be compatible and stable with all commonly used detergent compounds such as surfactants which mainly present in any detergent formulation (Kamini et al., 2000). The *S. arlettae* lipase was tested for its potential as an additive in detergents. The enzyme showed excellent compatibility and stability in the presence of ionic and nonionic surfactants as well as in commercial detergents (Fig.4.1). The enzyme showed increased stability in

presence of SDS, Tween 80 and similar results were reported for lipases from *Aspergillus sp*. and *Rhizopus sp* (Saisubramanian et al., 2006 and Derewenda et al., 1994). Among different detergents, wheel showed a maximum % increase in activity, while SDS exhibited an increase of approximately 6 % in activity over control (Fig.4.1). However, the lipase activity of *Ralstonia pickettii* (Hemachander and Puvanakrishnan, 2000) and *Aspergillus carneus* were inhibited in the presence of SDS (Saxena et al., 2003) while the activity was increased in *H. lanuginosa* (Omar et al., 1987).

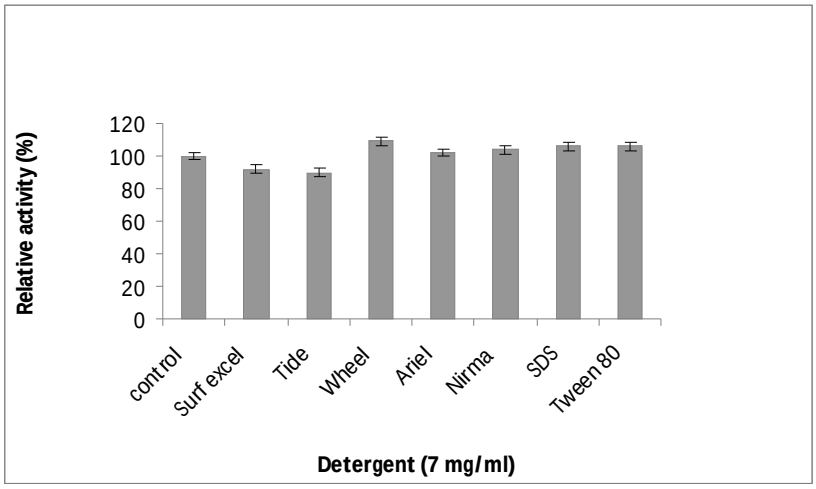

Figure 4.1: Compatibility of *S. arlettae* lipase with surfactants and detergents. For the control, lipase was incubated with buffer devoid of surfactants and detergents and its activity was taken as 100 %. All values are represented as mean $\pm$ s.d of three replications

4.3.2 Lipase compatibility with oxidizing agents

Bleach stability of the enzyme was also checked in the presence of hydrogen peroxide, sodium hypochlorite, sodium perborate and sodium peroxide. The lipase was highly stable towards oxidizing agents at 1.5 % concentration for 1 h at 30 ^{o}C and it retained 92 % of activity even at 2.0 % concentration of hydrogen peroxide, while activity was gradually decreased with increase in concentrations of sodium perborate and sodium hypochlorite from 1.0 to 2.0 % (Fig.4.2). Remarkably, the present lipase exhibited better resistance towards strong oxidizing agents especially hypochlorite (95 % activity at 1.0 % concentration) compared to the relative activity of lipolase (Novozymes, Denmark), which exhibited 43 % activity after 1 h treatment as reported by Rathi et al.,

2001. Therefore, the stability of oxidations is an important characteristic required for an enzyme to be incorporated into a detergent.

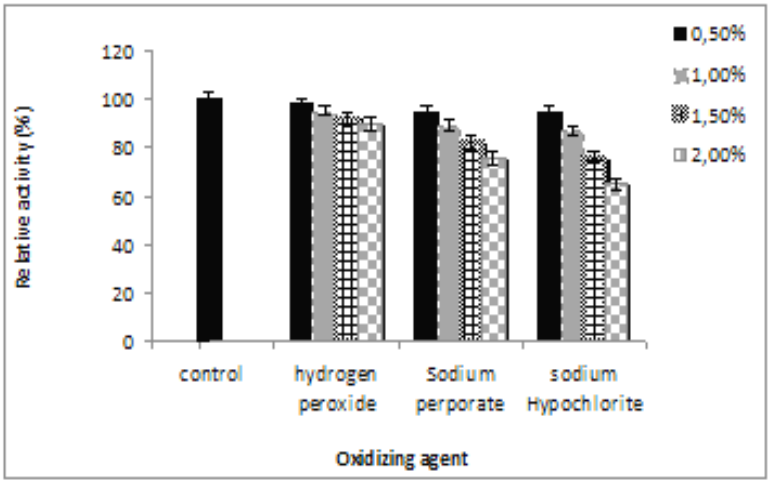

Figure 4.2: Compatibility of *S. arlettae* lipase with oxidizing agents. For the control, lipase was incubated with buffer alone without oxidizing agent and its activity was taken as 100 %. All values are represented as mean ± s.d of three replications

4.3.3 Effect of detergent and its concentration on oil removal

Effect of different commercial detergents on oil removal has been shown in Table 4.2. Among all detergents (0.3 %), wheel exhibited highest oil removal (52 %) from soiled cotton fabric and it has been chosen for subsequent studies. In detail, the lipase was more efficient with the nonionics than with the anionics. This is because the activity of the lipase is less inhibited by the nonionics than by the anionics (Sajna et al., 2012 and Flipsen et al., 1998). The relation between the concentration of surfactant in the presence of the lipase and the removal of olive oil is shown on Fig.3. At any concentration of any wheel detergent, the removal of olive oil with solution B-D-L was always higher than the solution B-D. Thus, it was proven that the lipase was effective with any detergent system at any concentration.

Table 4.2: Effect of lipase on removal of olive oil from cotton fabric with various detergents

Detergent	Oil removal (%) B^a+D^b	$B^a+D^b+L^c$
Tide	39.0	50.2
Run magic	33.2	45.5
Surf excel	36.4	47.5
Ariel	31.2	43.0
SDS	30.6	39.5
Tween 80	28.3	38.0
Wheel active	41.5	52.0
Nirma	30.2	41.2

[a] Buffer; [b] Detergent; [c] Lipase

It is proven, based on results of this study, at 0.5 % concentration of wheel that the lipase from *Staphylococcus arlettae* improves the removal of olive oil from cotton fabric by 37 (B +D) to 49 % (B+D+L) under the conditions of 30 units as the lipase concentration, 37 °C as washing temperature and 30 min of longer as washing time (Fig. 4.3). It can be expected that the lipase will be used for laundry detergents.

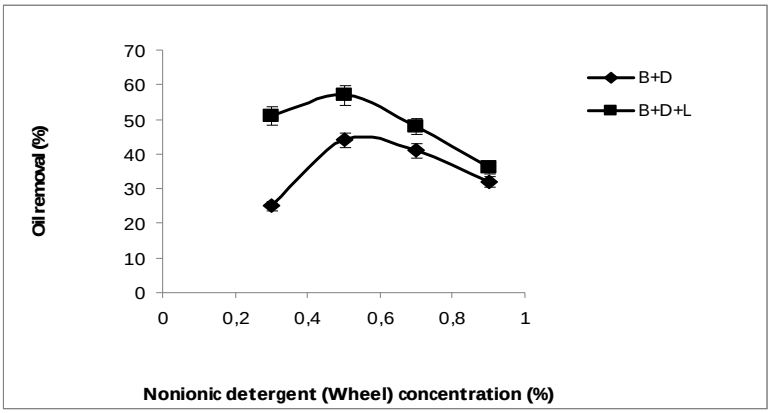

Figure 4.3: Effect of detergent and its concentration on oil removal (%) (Experimental conditions: Lipase amount 30 U; Washing temperature 37 °C; Washing time 30 min). All values are represented as mean ± s.d of three replications

4.3.4 Effect of lipase amount on oil removal

The relation between the lipase concentration and olive oil removal from stained fabric has been depicted in Fig. 4.4. In both cases, oil removal increases with lipase concentration till attaining the equilibrium state at a concentration of more than 40 units. The equilibrium attainment after certain lipase concentration has been depends on

the initial rate of hydrolysis of triglyceride by lipase based on the interface area between insoluble triglyceride and the aqueous solution of lipase. The surface area of a given amount of olive oil will be constant after a certain concentration of lipase with which interface is saturated. As shown in Fig.4, the addition of the lipase brought an improvement from 26 % to 55 % without the detergent and with detergent. Enhanced results of lipase in combination with a commercial detergent has been reported in several instances such as usage of *Pseudozyma sp*. NII 08165 (Sajna et al., 2012) produced biosurfactants and *Pseudomonas aeruginosa* lipases (Grbavcic et al., 2011).

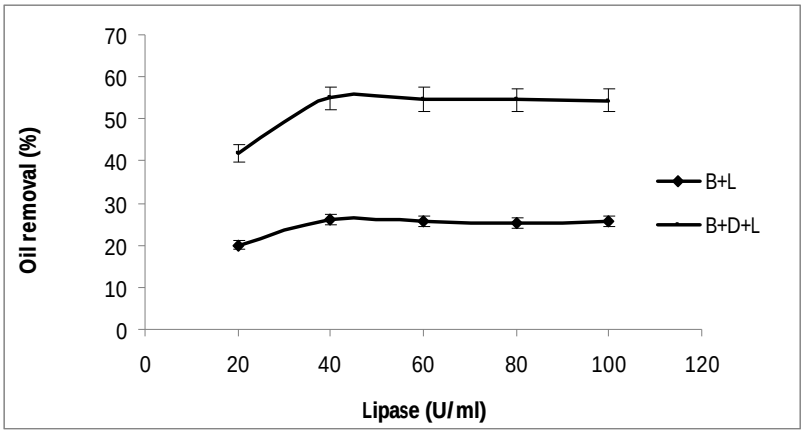

Figure 4.4: Effect of lipase amount on oil removal (Experimental conditions: Detergent concentration 0.5 %; Washing temperature 37 °C; Washing time 30 min). All values are represented as mean ± s.d of three replications.

4.3.5 Effect of washing temperature on oil removal

The results of oil removal with and without lipase at different temperatures have been shown in Fig 4.5. A maximum oil removal in case of B-L as a washing solution has been noticed at 37 ^{o}C. In the case of B-D washing solution, it has been seen that better oil removal has been takes place at higher washing temperature only (Horchani et al., 2009 and Romdhane et al., 2010). With solution B-D-L containing the wheel and the lipase, the removal was higher at any washing temperature than with any other solution. However, the contribution of the lipase to the removal of olive oil has been seen most significantly at the optimum temperature of the lipase of 40 ^{o}C.

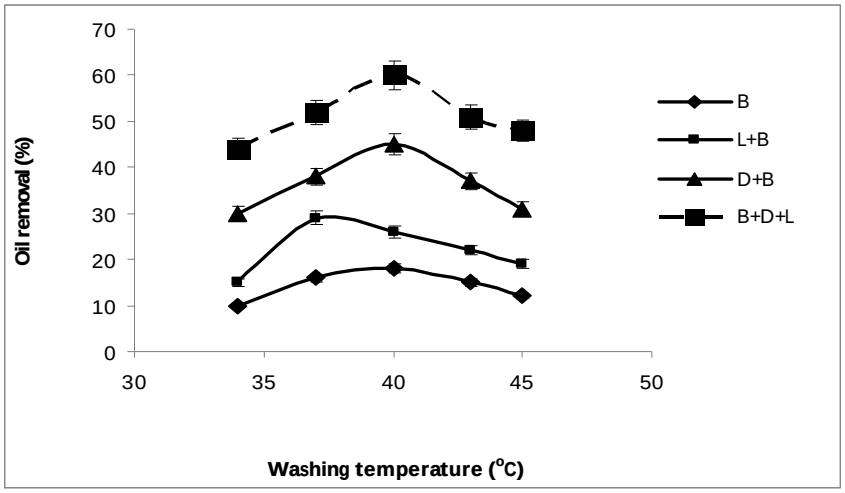

Figure 4.5: Effect of washing temperature on oil removal (Experimental conditions: Detergent concentration 0.5 %; Lipase amount 40 U; Washing time 30 min). All values are represented as mean ± s.d of three replications.

4.3.6 Effect of washing time on oil removal

The effect of washing time on oil removal has been depicted in Fig. 4.6. It has been observed that, on longer washing time, only B-L solution worked properly for oil removal from stained cloth. On the other hand, the removal with solution B-D became nearly constant after 20 min. Finally, it has been observed that utilization of B-D-L solution results with enhanced oil removal (62 %) in 45 min of washing cycle. Thus, the significant contribution of lipase in oil removal has also been observed with prolonged washing time. Sajna et al., 2012 has been reported that the enhanced stain removal was seen with increased wash time using *Pseudozyma sp.* lipase as a detergent additive. Similar enhancement with increasing wash time has also been reported by Grbavčić et al., 2011 in case of *Pseudomonas aeruginosa* lipase biodetergent study.

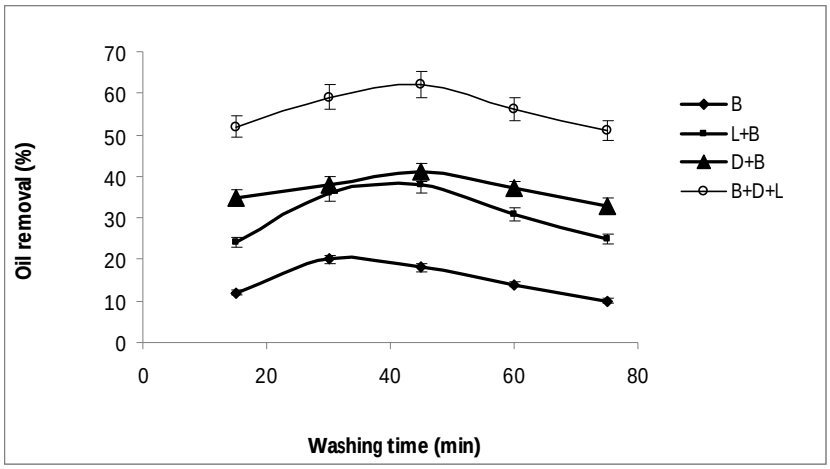

Figure 4.6: Effect of washing time on oil removal (Experimental conditions: Detergent concentration 0.5 %; Lipase amount 40 U; Washing temperature 40 °C). All values are represented as mean ± s.d of three replications.

4.4 CONCLUSIONS

Lipase from *S. arlettae* JPBW-1 was an ideal candidate for use in laundry detergent formulations, since it possessed better stability with surfactants, commercial detergents, bleaching and oxidizing agents. It has been found that the lipase was more compatible with nonionic surfactant than the anionic counterpart, which makes it a novel lipase for further commercial utilization in detergent formulations. The results of this study show that the lipase from *S. arlettae* improves the removal of oil from cotton fabric by 21 % as an additive commercial detergent viz. 0.5 % Wheel under the optimum conditions of 40 U of lipase in Tris-HCl buffer (0.1M, pH 8.0) at 40 °C as washing temperature in a washing time of 45 min. Hence, lipase from *S. arlettae* has been the ideal choice for formulating an environmentally friendly detergent formulation with the objective of oil removing from soiled fabrics.

CHAPTER 5

Modeling embedded optimization strategy for formulation of bacterial lipase based bio-detergent

ABSTRACT

Lipase based detergent formulations are one of the viable solutions for chemical detergents that have health and environmental hazards to customers and society. In this study, the efficacy of *Staphylococcus arlettae* JPBW-1 lipase has been assessed for the oil removal as an additive in laundry detergent through modeling and optimization approach of response surface methodology integrated genetic algorithm. A three-level five-factorial central composite design was used to evaluate the interactive effects of process conditions viz. detergent concentration, lipase concentration, buffer pH, washing temperature and washing time on oil removal (%) from cotton fabric. The input space of validated response surface model (RSM) (R^2 value of 97.7 %) has been utilized for the genetic algorithm (GA) optimization. The maximum oil removal of 79.6 % has been achieved with the GA predicted optimum values of 0.69 % detergent, 47.37 U of lipase, buffer pH of 7.2 and washing temperature of 37.18 ^{o}C in 26.11 min, which was 27 % more than the oil removal without lipase. Hence, lipase from *S. arlettae* JPBW-1 could be effectively used as an additive in laundry detergent for the oil removal from the soiled fabric and introduces a new lipase to the bio-based detergent industry.

5.1 INTRODUCTION

World's detergent market has been flooded with various brands with a main focus of attracting customer's by claiming the brighter and fragrance attributes of detergent irrespective of the health consciousness of the customers (Lomax et al., 1997). The annual consumption of detergents in India ranges to hundreds of thousands of tonnes, with a probable investment sector for different companies in the Indian arena (Ramachandran, 2000). Usage of chemical based phosphate cleansers and fragrances in detergent formulation results, in health problems in customers and water pollution (Basketter et al., 2012). It is wise to reduce the amount of phosphate based cleansers by replacing with bio-detergent formula based on the usage of industrial enzymes such as lipase and protease by minimizing the chemical based ingredients in the formulation (Saeki et al., 2007).

Detergent enzymes account for about 32 % of the total lipase sales and represent one of the largest and most successful applications of modern industrial biotechnology (Novozymes, 2007). Usually removal of triglycerides in laundry is done by using the alkaline solution of surfactant. During this operation, only free fatty acids were removed from the fabric by forming water soluble fatty acid soaps, but triglycerides were not saponified by the alkaline solution and will remain on the fabric itself. Addition of lipases to detergent formulations facilitates easy removal of triglycerides from the soiled fabric as lipase will aid in the degradation of triglycerides into free fatty acids, di and mono-acylglycerols, and possibly glycerol (Horchani et al., 2012). This step will also substantially reduce the usage of phosphate builders in detergent formulation; those are responsible for environmental problems and works at milder temperatures. Detergent formulations containing lipases need to withstand their activity under harsh conditions of temperature (30–60 ^{o}C), pH (9–12) and formulation adjutants such as surfactants and oxidizing agents (Ruchi et al., 2008). Detergent industries are relying mostly on recombinant lipases of *Thermomyces* sp. expressed in *Aspergillus oryzae* (Lipolase, Novozymes) and *Pseudomonas* sp. lipases for achieving these properties (Fernandez-Lafuente, 2010; Jaeger et al., 1994; Andualema and Gessesse, 2012). Jellouli et al., 2011 tested lipases from two *Bacillus* strains on artificial fat and protein dirt clothes in presence and absence of commercial powder detergent to investigate their cleaning effect. Lipases from *Candida cylindracea* (Fujii et al., 1986) and *A. niger* (Saisubramanian et al., 2006) were also reported for their olive oil removing efficiency from cotton fabric with soluble lipase. Hasan et al., 2006 reported 100 % stability of *Bacillus* sp. FH5 lipase at pH 10 and showed promising results when used in combination with different commercial detergents. Extremophilic cellulase, commercialized by Genencor was the first industrial extremozymes for use in textile detergents (Kottwitz and Schambil, 2005). Researchers are in continuous search for lipases which can withstand the harsh laundry detergent conditions such as detergents, oxidizing agents, alkaline pH, and high temperatures for successful application of these biocatalysts in the detergent industries.

Singh, 2012 stated that the modeling integrated optimization approach helps researcher in attaining optimal conditions of industrial process in less time and facilitates the understanding of the process. Now a day's statistical modeling integrated evolutionary optimization is widely accepted due to tackling of modeling and optimization tasks by overcoming the local optima problem, usually encountered in unparalleled search optimization approaches (Garlapati and Banerjee, 2010). Response surface Methodology (RSM) is a statistical tool used for experimental design, model building, factor effects evaluation and analyzing optimum conditions of factors for desirable responses (Bezerra et al., 2008). In previous studies of lipase applicability in detergent industries, some researchers utilized response surface methodology for attaining the optimal washing conditions for removal of triglyceride molecules from cotton fabrics using lipase of *Cryptococcus* sp. S-2 and *Lactococcus lactis* (Thirunavukarasu et al., 2008 and Uppada et al., 2012). In the present study we have utilized artificial intelligence based genetic algorithm for optimization purpose by using the input space of the RSM model (Goldberg, 2002). Genetic algorithm invented by John Holland is a search algorithm works on notions of natural phenomenon by imitating Darwin´s natural evolution (Zang et al., 2010). Hence, in the present study, we have made a first attempt to model and optimize the efficacy of extremophilic lipase (Chauhan and Garlapati, 2013) as an additive in laundry detergent for removal of olive oil from soil fabric through RSM integrated genetic algorithm.

5.2 MATERIALS AND METHODS

5.2.1 Microorganism

Staphylococcus arlettae JPBW-1 was used for the lipase production, which was isolated from the one and only hot spring of India, Darang, HP and deposited in MTCC, Chandigarh as *Staphylococcus arlettae* JPBW-1 MTCC5589, maintained on Luria agar slants at 4 ^{o}C.

5.2.2 Materials

Triton X-100 and *p*-nitrophenyl palmitate (*p*-NPP) were purchased from Sigma Chemical Co. (St Louis, USA). Detergent "Wheel active" was used of Procter and Gamble Home Products Ltd. Brand. Olive oil used was the brand of Sos Cuetara, S.A. Figaro. All other chemicals used were of analytical reagent grade and commercially available.

5.2.3 Preparation of enzyme solution

Lipase production was carried out by seeding the 9.3 % of inoculum in Erlenmeyer flasks (250 mL) containing 100ml LB medium supplemented with 10.28 % of soybean oil as an inducer. The flasks were incubated at 35 °C under agitation (100 rpm) for 3 h. (Chuahan and Garlapati, 2013). The culture was centrifuged at 5367 g for 15 min at 4 °C and the supernatant was partially purified by ammonium sulphate precipitation

5.2.4 Lipase assay

The lipase activity was estimated using a spectrophotometric method with *p*-NPP as a substrate (Garlapati and Banerjee, 2010). One unit of lipase activity was defined as the amount of enzyme that liberates 1 mmol of *p*-nitrophenol per minute under the standard assay conditions.

5.2.5 Preparation of soiled fabric and washing solution

Triglycerides soiled fabric were prepared and olive oil was selected as a model substrate as reported in earlier studies. Defatting of the cotton fabric (5 cm × 10 cm) was done by boiling in chloroform for 4 h and soiled by spotting 0.5 ml of olive oil in benzene (100 mg/ml concentration) twice with a micropipette. The washing solutions (B/BL/BD/BDL) were prepared as shown in Table 5.1.

Table 5.1: Composition of the washing solution

Components	**Volume(ml)**			
	B[a]	**BL[b]**	**BD[c]**	**BDL[d]**
Tris HCL (0.1M, pH8.0)	40	40	40	40
Detergent (0.5%)	-	-	50	50
Lipase (50U/ml)	-	10	-	10
Distilled water	60	50	10	-

B[a] : buffer; BL[b] : Buffer+ lipase ; BD[c]: Buffer+ Detergent; BDL[d] : Buffer+Detergent+ Lipase

Solution BDL contained buffer and the detergent solution, pre-incubated at 37 ^{o}C for 10 min to which lipase solution (50 U) was then added. Oil removal was considered as the dependent output variable and the data on percent oil removal obtained from RSM (Table 5. 2) were subjected to the analysis of variance (ANOVA).

5.2.6 Washing procedure and determination of olive oil

Soiled fabrics were washed at 37 °C by shaking at 100 rpm for 30 min. After that, the fabrics were rinsed thrice with 100 ml of water, each for a period of 2 min and then air-dried. Extraction of olive oil from the soiled fabrics was done by using petroleum ether (bp 40–60 ^{o}C) as a solvent in a soxhlet extractor for 6 hr. Then petroleum ether was completely evaporated and the weight of olive oil was determined. The removal of olive oil was calculated by the following equation based on the weight of olive oil before and after washing.

$$\text{Oil removal (\%)} = W_b - W_a/W_b \times 100 \quad \text{---------} \quad \text{Eqn (5.1)}$$

Where W_b is the weight of olive oil before washing and W_a is the weight of olive oil after washing.

5.2.7 Factorial design and data analysis

A three-level five factorial central composite design (CCD) was employed for studying the effect of individual and interaction effects on the oil removal.

Table 5.2: Experimental range of variables for the central composite design in terms of actual and coded factors

Variables	**Symbol coded**	**Range of variables**		
		Low (-2)	Mid (0)	High (+2)
Detergent concentration (% W/V)	A	0.3	0.5	0.7
Enzyme amount (U)	B	20.0	40.0	60.0
Washing temperature (^{o}C)	C	34.0	37.0	40.0
Buffer (pH)	D	7.0	8.0	9.0
Washing time (min)	E	15.0	30.0	45.0

Five independent variables, including detergent (Wheel active) concentration (A, 0.3–0.7 %), lipase concentration (B, 20–60 U), washing temperature (C, 34– 40 ^{o}C), buffer pH (D, pH 7–9), and washing time (E, 15-45 min) were studied at three levels, based

on preliminary results on removal of olive oil from cotton fabric with different detergents.

5.2.8 GA based optimization approach

Once the RSM model with good prediction accuracy is developed, a genetic algorithm can be used to optimize the input space *(*X*)* representing process variables, with an objective to maximize the process performance such as oil removal (%) from soiled fabric cloth. The input variables of RSM model serve as decision variables for GA. The decision variables chosen in this study are detergent concentration, lipase concentration, washing temperature, buffer pH and washing time. The sequence of events under GA optimisation are random creation of population, evaluation of the population based on the fitness value (Oil removal, %) and creation of a new population. A new population (reproduction) is created using the GA parameters of crossover (exchange of bits in a pair of chromosome) and mutation (flipping of bits in offspring). This process continues until an optimal solution is achieved (Zang et al., 2010). Under binary coded GA, real variables were encoded in form of binary character strings (1 and 0). One complete binary bit string was termed as a chromosome, composed of single bit strings where each single real value is encoded. Hence in the present optimization, a complete chromosome was composed of 50 bits, consisting of five process variables of 10 bit each. Stopping criterion was not pre-determined and was usually decided after evaluating the results over certain generations (Skolpap et al., 2008). The binary coded GA was executed through C language. In the present study, maximization problem was converted to minimization problem based on the duality principle with the purpose of optimizing the lipase based detergent washing for attaining the optimal oil removal (%) from the soiled fabric cloth by taking uncoded RSM equation as a basis.

5.3 RESULTS AND DISCUSSION

Lipases are the choice enzymes for detergent industry due to its degreasing ability of removing of oil and fat stains from soiled fabrics. An ideal detergent enzyme should be stable in presence detergent formulation ingredients such as surfactants, detergents and

oxidizing agents (Skolpap et al., 2008). Based on our previous laboratory studies, lipase from *S. arlettae* JPBW-1 showed high stability in presence of surfactants & detergents (with SDS, Tween 80, wheel) and oxidizing agents (with H_2O_2, Sodium hypochlorite and sodium perborate) (Chauhan et al., 2013). Lipase based detergent formulation is sensitive to many parameters such as detergent concentration, lipase amount, washing temperature, buffer pH and washing time (Cherif et al., 2011). In understanding the detergent formulation, particularly, bio based detergent formulation, modeling and optimization is a helpful tool for the assessment of individual and interaction effects of formulation expedients on final oil removal from the soiled fabric and for enhanced results (Sajna et al., 2013). RSM integrated GA strategy has been carried out with the aim of modeling and optimization of lipase based detergent formulation.

5.3.1 Model development, statistical analysis, and validation

Based on the results of our one variable at a time approach results, (Cherif et al., 2011) experiments were planned based on central composite design (CCD) of RSM taking five input variables of wash cycle viz. detergent concentration (A), lipase concentration (B), washing temperature (^oC), pH of the buffer (D) & washing time (E) and output as oil removal (%). The interactions among the factors played a more significant role than the effect of individual factors alone shown in many results (Rahulan et al., 2009).

The oil removal of *S. arlettae* JPBW-1 was expressed as a non-linear function of the input process parameters in coded form as follows:

$$OR\ (\%) = 74.4480 + 1.4889X_1 + 2.6611X_2 + 0.8389X_3 + 0.0333X_4 - 0.1611X_5 + 0.8062X_1X_2 + 0.3313X_1X_3 - 1.3312X_1X_4 + 0.5063X_1X_5 + 1.2437X_2X_3 + 0.0063X_2X_4 - 0.8312X_2X_5 + 1.4313X_3X_4 - 0.3062X_3X_5 + 1.3063X_4X_5 - 0.891X_1^2 - 4.641X_2^2 - 2.641X_3^2 + 2.509X_4^2 - 2.441X_5^2 \quad \text{----- Eqn (5.2)}$$

The predicted values were seems to be in close agreement with the experimental values after conducting the CCD experiments in triplicates (Table 5.3) which indicates the good prediction accuracy and generalization ability of the predicted model (Myers et al., 2009).

Based on the significance test results, the *P*-values (Table 5.4) of X_1, X_2, X_3 X_2^2 ,X_3^2,X_4^2,X_5^2,X_1X_2, X_1X_4,X_2X_3 X_2X_5.X_3X_4 and,X_4X_5, are found to be less than 0.05

(considering 95 % as a level of confidence) and are considered to have significant impact on the oil removal (%).

Table 5.3: Central composite design with the experimental and predicted responses.

	Input parameters					Response		
Run order	DC[a](%) (A)	EA[b](U) (B)	WTem.[c](^oC) (C)	pH (D)	WT[d] (min) (E)	OR[e](%) (Exp.)	(Predict.)	R-Studentized residual
1	0.5	40	37	7	30	76.3	76.9	-0.598
2	0.7	20	34	7	15	67.0	66.9	0.010
3	0.7	60	40	7	15	75.8	75.6	0.446
4	0.3	20	40	7	15	61.2	60.9	1.026
5	0.3	60	40	9	15	72.0	71.7	0.866
6	0.3	20	40	9	45	66.0	66.2	-0.777
7	0.7	60	34	7	45	70.0	70.1	-0.425
8	0.5	40	40	8	30	73.0	72.6	0.334
9	0.7	60	34	9	15	65.0	65.0	-0.123
10	0.5	40	34	8	30	71.0	70.9	0.029
11	0.7	60	40	9	45	74.2	74.3	-1.436
12	0.5	40	37	8	30	72.8	74.4	-1.246
13	0.5	40	37	8	30	73.0	74.4	-1.068
14	0.5	60	37	8	30	72.0	72.4	-0.444
15	0.7	40	37	8	30	76.5	75.0	1.588
16	0.7	20	34	9	45	64.0	64.4	-0.202
17	0.3	20	34	7	45	63.0	62.9	0.096
18	0.5	40	37	8	30	76.0	74.4	1.159
19	0.3	20	34	9	15	62.0	61.8	0.397
20	0.5	20	37	8	30	68.0	67.1	0.839
21	0.7	20	40	9	15	62.0	62.2	0.876
22	0.3	40	37	8	30	71.0	72.0	-1.080
23	0.5	40	37	9	30	78.0	76.9	1.012
24	0.3	60	34	7	15	68.0	67.5	2.142
25	0.5	40	37	8	15	71.2	72.1	-0.965
26	0.3	60	34	9	45	65.0	65.0	-0.037
27	0.7	20	40	7	45	63.4	63.7	-1.247
28	0.5	40	37	8	45	73.2	71.8	1.445
29	0.3	60	40	7	45	62.7	62.5	0.537

[a] Detergent concentration; [b]Enzyme amount: [c]Washing temperature; [d]Washing time and [e]Oil removal

Table 5.4: Results of significance test on the non-linear model-coefficients, standard errors, T statistics, and *P* values for the oil removal (coded form)

SI.no.	Terms	Coeffecient	Standard Error Coeffecient	T	P
1.	Constant	74.448	0.476	156.39	0.000
2.	X_1	1.488	0.341	4.365	0.002
3.	X_2	2.661	0.341	7.801	0.000
4.	X_3	0.838	0.341	2.459	0.039
5.	X_4	0.033	0.341	0.098	0.925
6.	X_5	-0.161	0.341	-0.472	0.649
7.	X_1^2	-0.891	0.924	-0.964	0.368
8.	X_2^2	-4.641	0.924	-5.022	0.001
9.	X_3^2	-2.641	0.924	-2.858	0.021
10.	X_4^2	2.509	0.924	2.715	0.026
11.	X_5^2	-2.441	0.924	-2.642	0.030
12.	X_1X_2	0.806	0.361	2.228	0.056
13.	X_1X_3	0.331	0.361	0.915	0.387
14.	X_1X_4	-1.331	0.361	-3.679	0.006
15.	X_1X_5	0.506	0.361	1.399	0.199
16.	X_2X_3	1.243	0.361	3.437	0.009
17.	X_2X_4	0.006	0.361	0.017	0.987
18.	X_2X_5	-0.831	0.361	-2.297	0.051
19.	X_3X_4	1.431	0.361	3.956	0.004
20.	X_3X_5	-0.306	0.361	- 0.846	0.422
21.	X_4X_5	1.306	0.361	3.610	0.007
SS = 1.447		R-Sq = 97.7%		R-Sq(adj) = 92.1%	

Significant contribution of the linear, square & interaction terms towards the response and regression model have been seen through ANOVA results (Table 5.5) through their *P*-values, which were less than the significance level (α 0.05) for all the terms and through the *F*-value of 17.25, a very low probability value (< 0.0001). The coefficient of multiple regression, R^2 values and the adjusted R^2 values was found to be 97.7 % and 92.1 % respectively, which indicate the fitness and adequacy of the model. Non-significant lack of fit (0.54) indicates the model equation has been adequate for

predicting the percent of oil removal from soiled fabric cloth under any combination of values of the variables (Kishore and Kayastha, 2012).

Table 5.5: ANOVA for the quadratic model for percent oil removal

Source	DF	Sequential SS	Adjusted SS	Adjusted SS	F	P
Regression	20	722.784	722.784	36.139	17.25	0.000
Linear	5	180.524	180.524	36.105	17.24	0.000
Square	5	400.264	400.264	80.053	38.22	0.000
Interaction	10	141.996	141.996	14.200	6.78	0.006
Residual Error	8	16.757	16.757	2.095		
Lack of Fit	6	10.331	10.331	1.722	0.54	0.766
Pure Error	2	6.427	6.427	3.213		
Total	28	739.541				

The interaction effects of detergent formulation variables on % oil removal have been further confirmed through the response surface plots, depicted in Fig. 5.1. The cumulative effect of detergent concentration with enzyme amount, washing temperature and washing time respectively on oil removal has been shown in Fig.5. 1(A), (B) & (C). In each case, higher oil removal (%) from soiled fabric cloth attained at near the +1 level and later, the decreasing oil removal values have been noticed. The convex shape of response surface plots in Fig. 5.1 (D), (F) and (H) shows the significant interaction of enzyme amount with washing temperature & washing time and washing temperature & washing time on oil removal. In these plots, maximum oil removal (%) has been achieved nearby mid value (0) level. Maximum oil removal (%) from the soiled fabric cloth has been obtained at higher buffer pH (Fig. 5.1 E & G). The response equation can be written in the uncoded form as follows:

OR (%) = - 47.7723+49.4181(A) + 0.274126 (B)+ 17.2770 (C) -57.0592 (D)+ 0.221798 (E) + 0.201562 (A)(B) + 0.552083 (A((C) -6.65625 (A)(D)+ 0.168750 (A)(E) + 0.0207292 (B)(C) + 0.000312500 (B)(D) - 0.00277083 (B)(E) + 0.477083 (C)(D) - 0,00680556 (C)(E) + 0.0870833 (D)(E) -22.2757 (A^2) -0.0116026 (B^2) - 0.293448 (C^2) + 2.50897 (D^2) – 0.0108490 (E^2) --------- Eqn. (3)

Where A, B, C, D and E represents the input process parameters.

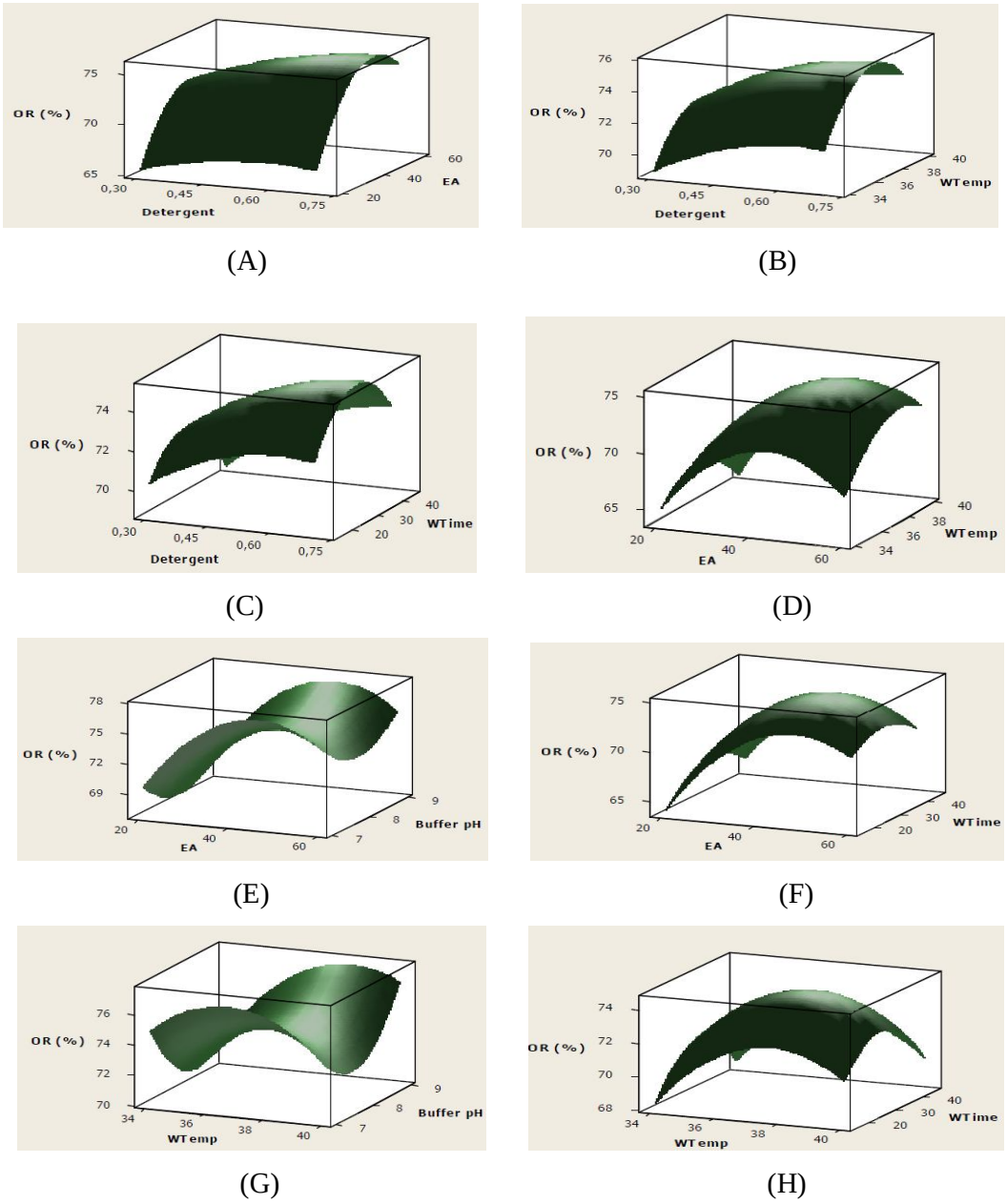

Figure 5.1: Surface plots of oil removal (%) with: (A) Detergent (% w/v) and Enzyme amount (U/ml), (B) Detergent (% w/v) and Washing Temperature (^{o}C), (C) Detergent (% w/v) and washing time (min), (D) Enzyme activity (U/ml) and Washing Temperature (^{o}C), (E) Enzyme activity (U/ml) and Buffer pH, (F) Enzyme activity (U/ml) and Washing time (min), (G) Washing temperature (^{o}C) and Buffer (pH), (H)Washing temperature (^{o}C) and washing time (min).

An optimized oil removal of 77.02 % using 0.68 % w/v Detergent, 60 U lipase and buffer pH of 7.2 in 20 min at 36.66 ^{o}C predicted by the response surface optimizer of MINITAB 14 software. The predicted variables were chosen for the triplicate experimental sets for lipase production, which resulted in the oil removal of 76.27 % with close agreement with the predicted oil removal (77.02 %) with a % deviation of -0.97. Similarly enhanced results have also been reported in case of *Pseudomonas aeruginosa* and *Talaromyces thermophilus* lipase based oil removal from soiled fabric cloth using statistical based RSM (Sarabia and Ortiz, 2009; Grbavčić et al., 2011).

5.3.2 Optimization through binary coded GA

In this study, the input space of the significant uncoded RSM model have been taken as the fitness function for binary coded GA optimization and has been executed as minimization problem (based on the duality concept). The optimization search has been initially started with the standard values of 50 chromosome length (l_{chr}) and crossover probability (P_{cr}) of 0.5 for selection of mutational probability (*Pm*). The search has been executed in the range of 0.0001-0.0041 and results in maximum fitness value at 0.0001 *Pm*. The search further has been conducted for optimum value for GA parameters namely Population size (10-330) and Number of Generations (5-1200). The parametric results of the binary coded GA have been shown in Fig. 5.2, and the best individual result i.e 79.64 % oil removal, has been obtained in 978 generations (in a tested range of 1200) with 100 population size using a 0.0001 mutation probability. The GA predicted detergent formulation conditions were seems to be equal to detergent concentration of 0.69 % w/v, 47.37 U lipase and buffer pH of 7.0 in 26 min at 37.18 ^{o}C.

5.3.2.1 Verification of binary coded GA predictability

The optimum conditions for oil removal from soiled fabric cloth were obtained through RSM integrated GA optimization approach and were also used to validate experimentally and predict the values of the responses using the model equation. The result showed that experimental value (79.6 %) was reasonably close to the predicted value (79.64 %) with no significant difference with enhanced oil removal (more than 2.6 %) than the RSM optimized value (77.02 %).

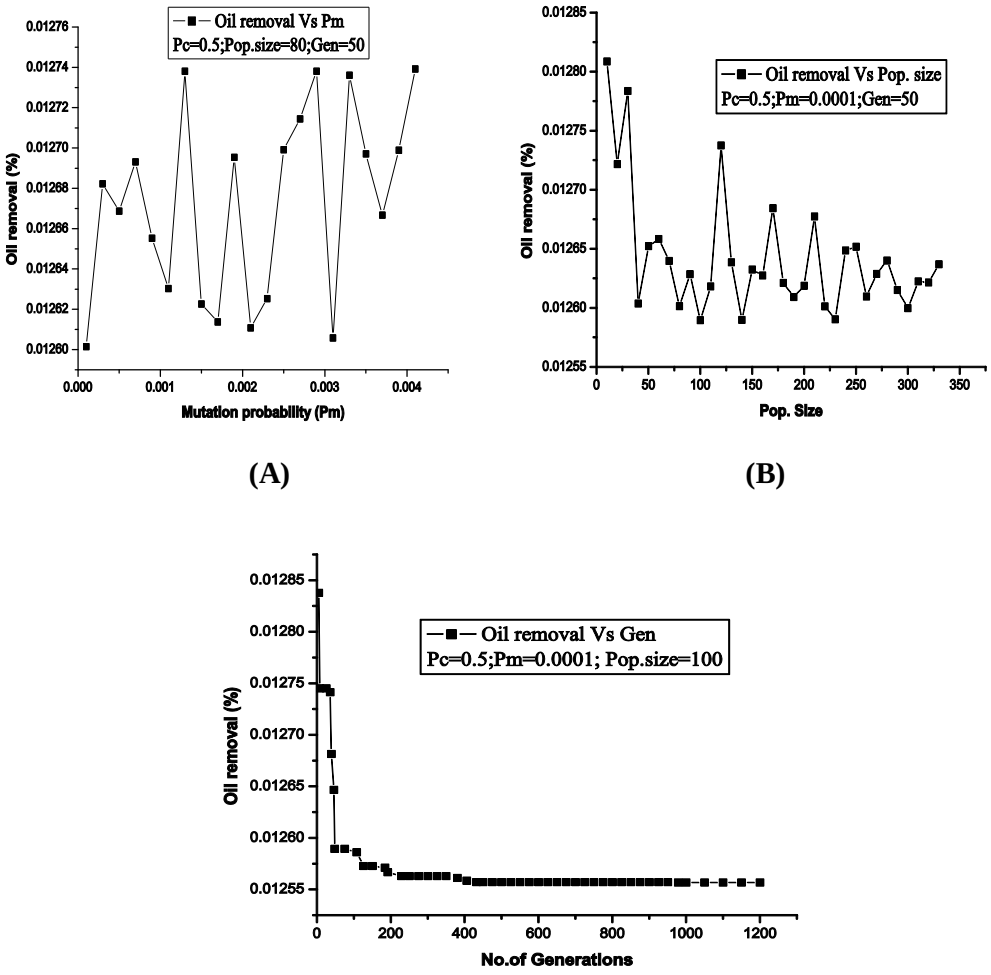

Figure 5.2: Parametric study of GA. (A) Oil removal Vs Mutation probability (Pm) (B) Oil removal Vs Population size (C) Oil removal Vs Maximum number of generations.

The superiority of GA over RSM lies as RSM assumes only quadratic non-linear correlation based local optima value using gradient based approach (Romdhane et al., 2010; Zafar et al., 2012). On the other hand, non- gradient based GA may find a design near to the global optimum within a reasonable time and computational costs and overcomes the local optima limitation of RSM. Moreover, the gradient methods such as

statistical and numerical optimization approaches use single points in search space whereas GA maintains a population of potential solutions. The superiority of GA over RSM has also acknowledged by several researchers in bioprocess area (Garlapati and Banerjee, 2010 and Skolpap et al., 2008). Based on the ideal characteristic of detergent - enzyme (effective at very low concentrations), the GA proposed lipase from *S. arlettae* was effective in removing the olive oil from the fabric using low concentrations (47 U) than the RSM predicted lipase concentration (60 U). Similar results of working under low concentrations were also reported in case of *C. cylindracea* and *R. pickettii* lipases for the removal of olive oil from soiled fabric (Fujii et al., 1986 and Hemachander and Puvanakrishnan, 2000). Hence, the halophilic, solvent tolerant lipase from *S.arlettae* could be effectively used as an additive in laundry detergents.

5.4 CONCLUSIONS

Statistical integrated artificial intelligence approach has been successfully implemented to study the modeling and optimization of lipase additive capability in the detergent formulation, which facilitates the modeling and subsequent optimization of the process. Binary coded GA based on RSM validated model (R^2 value of 97.7 %) has been used for optimization of washing process using lipase as additive, led to removal of 79.6 % oil from soiled fabric cloth with the GA predicted optimal variables of 0.69 % detergent, 47.37 U of lipase, buffer pH of 7.2 and washing temperature of 37.18 ^{o}C in 26.11 min. The optimization ability of GA has shown its superiority over RSM with comparative less value of oil removal (%) from soiled fabric cloth. Hence, the proposed approach of integrating statistical modeling with GA showed the potential in optimizing the process and will be suitable for optimizing the productivity of different industrial biotechnological processes.

CHAPTER 6

Purification of *Staphylococcus arlettae* lipase

ABSTRACT

An extracellular thermostable lipase was purified to homogeneity by using anion exchange chromatography and gel-filtration chromatography using DEAE-Sepharose and Sephacryl S-200 columns, respectively, with 27 fold purification and 32.5 U/mg specific activity. The purified enzyme showed a prominent single band on SDS–PAGE. The molecular weight of the purified enzyme was estimated to be 45 kDa. Based on matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometer (MALDI-TOF-MS) identified an amino acid sequence which shared similarity with α/β hydrolase fold gi|427702968 *Cyanobium gracile* PCC 6307.

6.1 INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are one of the most important classes of hydrolytic enzymes that catalyse both the hydrolysis and the synthesis of esters. Moreover, bacterial lipases are preferential enzymes for a wide range of industrial reactions due to its catalytic activity in aqueous and non-aqueous reactions. Only about 2 % of world's microorganisms have been tested as enzyme sources until 2006, and lipases from different sources have large variations in enzymatic activity, fatty acid specificity, optimal temperature, and pH (Hasan et al., 2006). Lipases with molecular weight range of 19–60 kDa belong to the α/β hydrolase family. The active site is formed by a catalytic triad of Ser, Asp/Glu and His. The active site of lipase is covered by a lid-like α-helical structure. The lid moves away upon binding to a lipid interface, causing the active site of lipase fully accessible, enhancing hydrophobic interaction between the enzyme and lipid surface (Jaeger and Reetz, 1998). Extracellular lipases have been proven to be efficient and selective biocatalysts in many industrial applications such as biosensors, pharmaceuticals, foods, cosmetics, detergents (Pandey et al., 1999). Lipases differ from one another by size, substrate specificity, stability profile, and activity in the presence of various activators and inhibitors. Given the importance of lipases in various industrial applications, there is much interest in isolating novel enzymes from unique environmental niches. Although lipases can be obtained from plants and animals, microbial lipases possess useful features such as high

yield and low production cost, diversity in catalytic activities, amenability to genetic manipulation, stability in organic solvents and broad substrate specificity (Shu et al., 2010). While a large number of different lipases have been discovered and commercialized (Hasan et al., 2006), new lipases with better characteristics are desirable, such as high activity and stability in non-aqueous media, (Xiao et al., 2009 and Bisen et al., 2010), and stability under alkaline conditions and in the presence of surfactants for use in detergent formulations (Hasan et al., 2010). Lipases have been purified from animal, plant, fungal and bacterial sources using variety of methods involving ammonium sulphate precipitation, ion exchange chromatography followed by gel filtration. Since industrial processes are commonly carried out under harsh conditions, it would be of great importance to obtain lipases which retain their optimal activity at extremes of temperature, pH, different concentrations of salts and in the presence of organic compounds normally used in the industrial reactions as solvents (Mellado et al., 2005). In this sense, the lipases isolated from extreme microorganisms constitute an excellent alternative in the industrial processes (Pikuta et al., 2007). Furthermore, the purified peptides were applied to the matrix-assisted laser desorption ionization- time of flight tandem mass spectrometer (MALDI-TOF/TOF MS) to determine their molecular mass and their amino acid sequence.

6.2 MATERIALS AND METHODS

6.2.1 Chemicals and reagents

p-Nitrophenyl palmitate, sodium dodecyl sulphate (SDS) and gel filtration markers, coomassie brilliant blue R-250 bromophenol blue, phenylmethyl-sulfonylfluoride (PMSF), DEAE sepharose and sephacryl S-200 were obtained from Sigma Chemical Company, USA. All other chemicals used were of analytical grade and of the highest purity available locally.

6.2.2 Microrganism and lipase production

The bacterial strain, *Staphylococcus arlettae* JPBW-1 used in this study was isolated from a rock salt mine Darang HP, India and identified by 16S rRNA analysis.

Staphylococcus arlettae was cultured in L.B medium (10 g of casein enzymic hydrolysate, 5 g of yeast extract, 10 g sodium chloride per litre) (pH 7). Submerged fermentation was carried out by seeding the spore suspension (50ml) in Erlenmeyer flasks (1000 mL) containing 500 mL of the L.B medium ,supplemented with soyabean oil (12 % v/v). The flasks were incubated at 35 °C under agitation (100 rpm) for 3 h. After incubation the fermentation medium was harvested by centrifugation at 6314 g for 10 min at 4 °C (Chauhan and Garlapati, 2013). The supernatant was collected and subjected to estimate the lipase activity.

6.2.3 Lipase assay and protein estimation

Lipase activity was assayed quantitatively using *p*-nitrophenyl palmitate as the substrate according to the method described by Garlapati and Banerjee, 2010. One enzyme unit was defined as the amount of enzyme that liberated 1 μmol of 4-nitrophenol per minute under the assay conditions. Protein content of cell-free supernatant was determined according to modified Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard.

6.2.4 Purification of *S. arlettae* lipase

6.2.4.1 Ammonium sulphate precipitation

The cell-free culture supernatant was precipitated using solid ammonium sulphate to 60 % saturation (Chauhan and Garlapati, 2013). The pellet obtained after centrifugation (30 min at 8000 rpm) was dissolved in 10 ml of buffer (50 mM Tris HCL pH 8). Insoluble material was removed by centrifugation at 8000 rpm during 5min. This is subjected to dialysis against 50 mM Tris–HCl buffer, pH 8 overnight.

6.2.4.2 Ion exchange chromatography

The dialyzed material was directly applied on a DEAE-Sepharose column (15×1.6 cm i.d.), previously equilibrated with 50mM Tris–HCl buffer, pH 8. The exchanged material was eluted with a linear gradient of NaCl ranging from 0.0 to 0.5M prepared in the same buffer at a flow rate of 60 ml/h and collected in 3ml fractions. Fractions coming under the peak were pooled and dialyzed and their activity was studied. The

fractions with high specific activity were then pooled and concentrated for further purification.

6.2.4.3 Gel Filtration chromatography

The concentrated enzyme was placed on Sephacryl S-200 column (93cm×1.6cm i.d) that had been equilibrated with 50mM Tris–HCl buffer, pH 8 and developed at a flow rate of 30 ml/h. Finally, 3ml fractions were collected screened for enzyme activity. Active fractions were pooled for subsequent analysis.

6.2.4.4 Molecular Weight Determination through SDS-PAGE

The molecular mass of the denatured protein was investigated using SDS-PAGE (Amresco, USA). A SDS-12.5 % polyacrylamide gel was prepared according to the method of Laemmli, 1970 with modification. Reference proteins for molecular mass determination (Biorad 161-0317) were lysozyme (14.4 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase 31 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), phosphorylase b (97.4 kDa) b-galactosidase (116 kDa) and myosin 200 kDa). Protein bands were visualized by silver staining.

6.2.4.5 Peptide mass fingerprinting by MALDI-TOF-MS

Mix sample and matrix solutions 1: 2 in the tube and spotted on the sample stage Load 0.5-1.0 µl of premixed matrix, allow the mixture to air dry until all solvent is evaporated, usually less than 5 minutes. It was dried at room temperature then washed with 0.1 % TFA and was analyzed by Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Mass spectrometer was calibrated by peptide calibration standard II (Bruker). Acquired mass spectra had a resolution of ~6000 (FWHM), which was sufficient to identify the digested peptide. The mass/charge spectra obtained were searched in MASCOT search engine (http://www.matrixscience.com) using all the 3 databases (MSDB, SwissProt, NCBInr). For search, peptides were assumed monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues, and peptide mass tolerance of 1.2 kDa was used for peptide mass fingerprinting.

6.3 RESULTS AND DISCUSSION

Enzyme purification is a series of processes intended to isolate a single type of enzyme from a complex mixture. Wide range of techniques are available and the choice depends on factors such as nature of the source i.e., extracellular or intracellular, scale of operation, stability of the enzyme etc. Success of enzyme purification scheme depends on the selection and effective combination of number of procedures. The objectives of such schemes are: high degree of purity, overall high recovery of enzyme activity and reproducibility of the process.

6.3.1 Purification and Molecular weight determination of lipase

The enzyme extract from *S. arlettae* has been subjected to a three-step purification scheme involving ammonium sulphate precipitation, ion exchange (DEAE sepharose) chromatography and gel filtration (Sephadex S-200). The enzyme extract was precipitated by ammonium sulphate for concentration. The precipitate was dissolved in minimum volume of tris buffer (pH 8.0) and dialysis was done in order to remove any salt concentration. Then it was loaded in the pre equilibrated DEAE - Sepharose column. Two major proteins peaks (A_{280}) was obtained in which most of the lipase activity was recovered in Peak 2 Fig. 6.1.

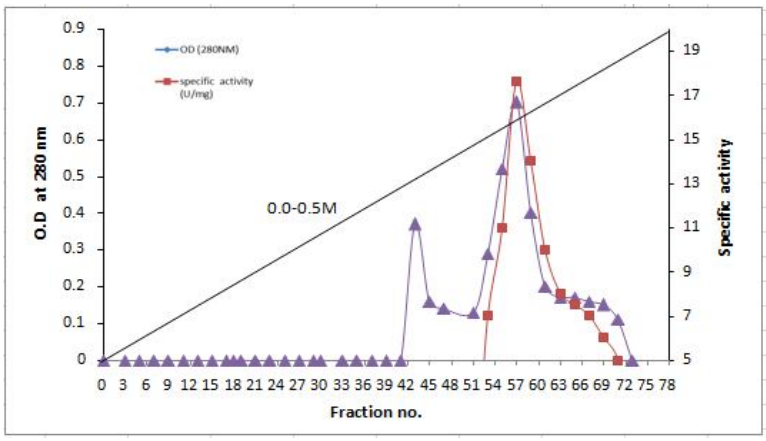

Figure 6.1: Elution profile of lipase from DEAE-sepharose column**.**

Lipase active fractions were pooled and concentrated. Gel filtration chromatography was followed after the ion exchange chromatography that facilitated salt removal and major impurities came while eluting the protein from the anion-exchanger column in the previous step. The protein was eluted as a single peak, the fractions were collected and concentrated (Fig 6.2).

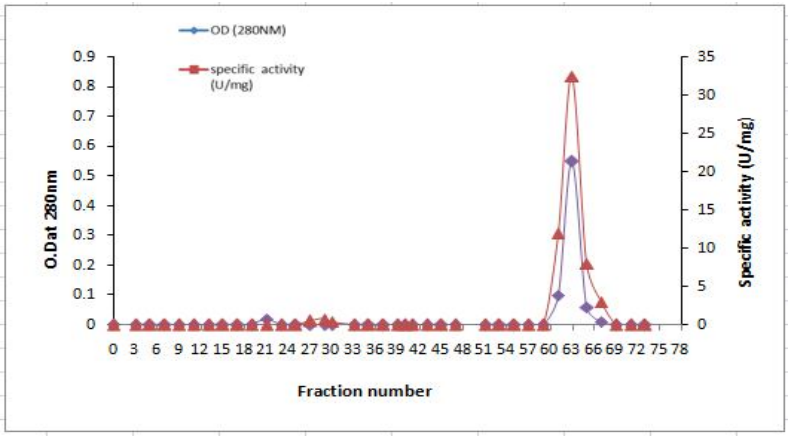

Figure 6.2: Elution profile of lipase from Sephacryl S-200 column

Lipase activity and protein concentration were measured. Finally, a homogeneous lipase with specific activity of 32.5 U/mg and 27-fold of purification with 11 % yield was obtained. Purification results are summarized in Table 6.1.

Table 6.1: Purification scheme of lipase from *S.arlettae*

Step	Total Units(U)	Total protein (mg)	Specific Activity(U/mg)	Purification fold	Recovery (%)
Crude enzyme	354.00	299.48	1.18	1	100
$(NH_4)_2SO_4$ precipitation	110	25	4.40	3.72	31.07
DEAE sepharose	88	5.2	17.6	14	24
Sephacryl S200	39	1.2	32.5	27	11

The eluent from the Sephacryl S-200 column yielded a single activity peak, and the protein was electrophoretically homogenous with a single band on SDS-PAGE. The purified lipase gave a single band of protein on 10 % SDS-PAGE (Fig. 6.3), indicating its homogeneity. A molecular weight of 45 kDa approximately has been estimated for

Staphylococcus arlettae. The molecular weight of *S. arelette* lipase was similar to that obtained from *Mucor hiemalis* (45 kda) (Hio et al., 1999) and *Staphylococcus aureus* lipase was estimated to be 45 kDa by SDS-PAGE (Xie et al., 2012).

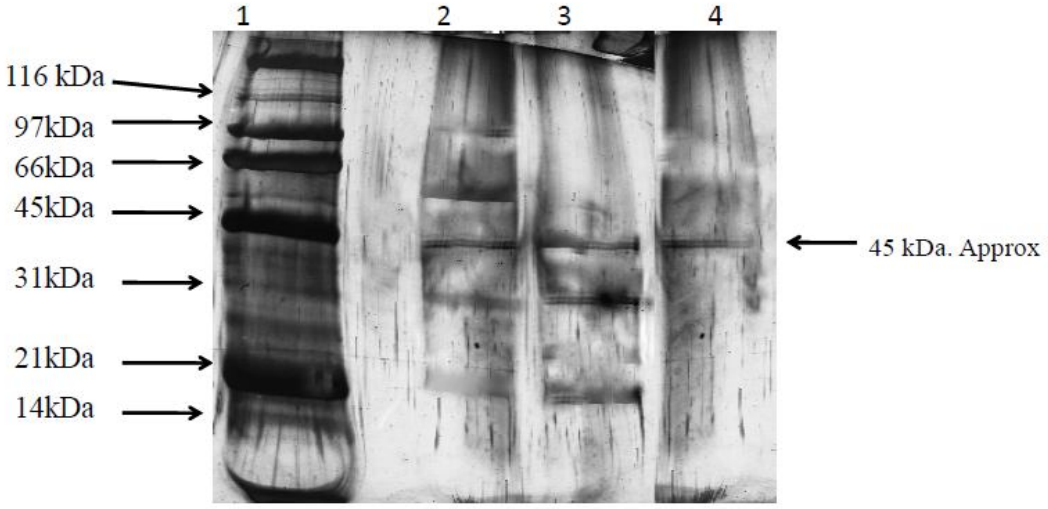

Figure 6.3: SDS-PAGE analysis on the purified enzyme. Lane 1: molecular weight markers; Lane 2: crude enzyme; Lane 3:Partially purified lipase (after DEAE-Sepharose column chromatography): Lane 4: Purified lipase (after Sephacryl S-200 column chromatography.

6.3.2 Peptide mass fingerprinting by MALDI-TOF-MS

Peptide-mass fingerprinting was carried out and data searched against NCBI databases showed that the peptide identified an amino acid sequence which shared similarity with α/*β* hydrolase fold gi|427702968 *Cyanobium gracile* PCC 630 covering 39 % of the entire sequence (219 amino acid residues). The MALDI-TOF peptide mass fingerprint (PMF) spectrum of fragments of lipase derived through trypsin digestion (in-solution).The sequence coverage of these fragments is shown in red, in the inset (Fig. 6.4). BLASTp analysis revealed similarities to other proteins Esterase/lipase *Prochlorococcus marinus* str. MIT 9303, Esterase/lipase *Prochlorococcus marinus* str. MIT 9313, Esterase/lipase *Synechococcus sp*. RS9916 provided 63 %, 64 % and 58 % identity respectively.

Conserved domains present in the encoded protein were also analysed using the NCBI Conserved Domain Search, which revealed a *α*/*β* hydrolase domain family. Bacterial lipases have a characteristic *α*/*β* hydrolase fold (Nardini et al., 1999). Homology was

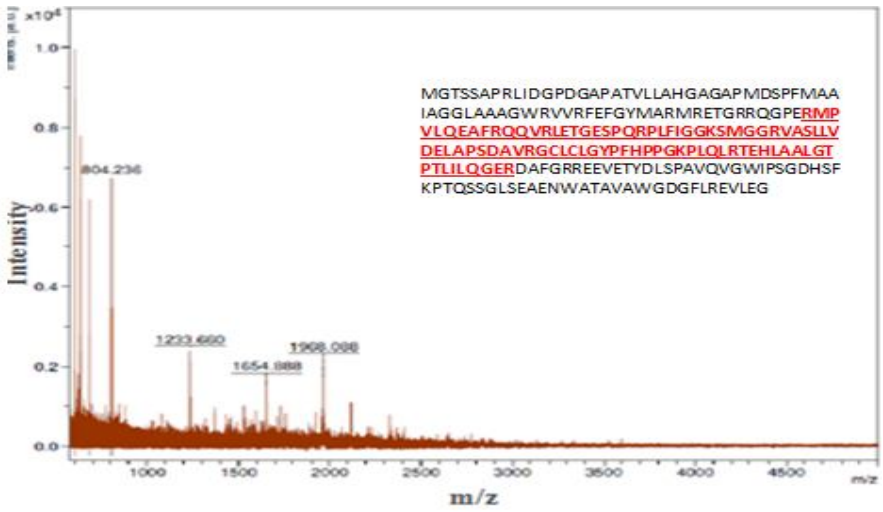

Figure 6.4: The MALDI-TOF-MS profile of the purified enzyme. The prominent mass peaks were screened using a database search for the peptide mass fingerprint with the MASCOT search program.

detected with lipase, the classical *α*/*β* hydrolase domain was identified between amino acids 137 and 185 which contains the putative catalytic serine. To verify that *α*/*β* hydrolase domain was indeed a serine hydrolase, a sample of enzyme was treated with the inhibitor PMSF which binds covalently to serine residues (Chauhan and Garlapati, 2013). Inhibition of activity with serine inhibitors shows that this lipase belongs to the class of serine hydrolases (Yadav et al., 1998 and Gilbert et al., 1991), which clearly indicated that a serine was involved in the catalytic mechanism.

6.4 CONCLUSIONS

In this study, the *S. arlettae* lipase was purified for the first time. The lipase was purified about 27 fold with molecular weight has been found to be 45 kDa approximately. MALDI-TOF-MS identified an amino acid sequence which shared similarity with α/*β* hydrolase gi|427702968 *Cyanobium gracile* PCC 6307.

Summary and Future prospects

SUMMARY

The research work carried out can be summarized in following points

- In the present study, *S. arlettae* was identified to produce a halo- thermo-solvent tolerant lipase. The fermentation process conditions were optimized for maximum lipase production using classical method of one factor at a time approach. The optimized parameters are 10 % inoculum with 12 % of soybean oil as carbon source utilizing a pH 8.0 in 3 h at 35 °C and 100 rpm through submerged fermentation.
- The characterization of partially purified lipase produced by *S. arlettae* showed stability in organic solvents, surfactants, such as Tween 80, Tween 40 and chelator EDTA. It remained active at 90 °C, pH 12 and upto 20 % NaCl. Enzyme activity was stimulated by Ca^{2+}, Hg^{2+} and Mn^{2}, and inhibited by K^{+}, Zn^{2+}, and Co^{2+}. Lipase kinetic studies were conducted using p-NPP as the substrate and the data obtained and from this, the K_m and V_{max} were recorded as 7.05mM and 2.67mmol/min respectively. These properties make this lipase an ideal candidate for industrial application from the perspectives of its tolerance towards industrial extreme conditions of pH, temperature, salt and solvent.
- The fermentations process parameters were further optimized using statistical based RSM and GA algorithm approach. A second order polynomial response surface model has been developed successfully and utilized in search of optimal conditions for lipase production through SmF using binary coded GA. The optimum fermentation conditions obtained for the synthesis of lipase from *S. arlettae* were 38.8°C, oil concentration 10.2%, inoculum volume 9.3%, pH 7.32, and incubation time 3 h for obtaining, a maximum lipase activity of 6.45 U/mL. An overall 1.8-fold increase in lipase activity was achieved after fermentation variables optimization, following the stastical approach.
- The compatability of the lipase was studied with surfactants, commercial detergents, bleaching and oxidizing agents. The results showed that lipase has

detergent, surfactants and oxidising agents stability added with alkaline nature and hence desirable for application in detergent functions. The results of this study show that lipase from *S. arlettae* improves the oil removal from soiled cotton fabric by 21% as an additive to commercial detergent, namely, 0.5% wheel under optimum conditions of 40U of lipase in Tris-HCl buffer (0.1 M, pH 8.0) as washing temperature and washing time seems to be equal to 40°C and 45 mins, respectively.

- The washing process parameters were further optimized using statistical integrated artificial intelligence approach. Optimization of washing process using lipase as additive by binary coded GA based on the validated the polynomial model of RSM (R^2 364 value of 97.7%). The predicted optimum process conditions using GA was found as of 0.69% detergent, 47.37 U of lipase, buffer pH of 7.2 and washing temperature of 37.18 ° C in 26.11 mins led to removal of 79.6 % oil from soiled fabric cloth.
- After optimization of process conditions using RSM and GA, the oil removal was enhanced by 1.5 fold than classical method of one factor at a time approach. The optimization ability of GA was found to be superior to that of RSM with a comparatively lower oil removal percentage from soiled fabric. Thus, this work demonstrated the feasibility proposed approach statistical modeling with integrated GA to develop optimum process conditions with a minimum number of experimental trials.
- In this study, the *S. arlettae* lipase was purified for the first time. The lipase was purified about 27 fold with molecular weight has been found to be 45 kDa approximately. MALDI-TOF-MS identified an amino acid sequence which shared similarity with α/*β* hydrolase gi|427702968 *Cyanobium gracile* PCC 6307.

FUTURE PROSPECTS

There is ample scope for further research on the biochemistry of the lipase, structure elucidation and enzyme engineering towards a wide range of further applications, besides enriching scientific knowledge on extremophilic enzymes.

- Commercialization of this new lipase based biodetergent formulation.
- Protein engineering studies for enhanced yield and catalytic properties.
- Purified lipase can be used for application of lipases in the leather, paper & pulp industries.
- Application of lipase in bioremediation for oil spills and bio-diesel production

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Appendices

Appendix A

Figure 2.1

Incubation temp. (°C)	Lipase activity (U/ml)
25	0.09987
30	0.10324
35	0.21556
40	0.09871
45	0.08432
50	0.0753

Figure 2.2

Inoculum size (%)	Lipase activity (U/ml)
2	0.03804
4	0.10144
6	0.18386
8	0.15216
10	0.3487
12	0.05072

Figure 2.3

Media pH	Lipase activity (U/ml)
4	0.06974
6	0.12046
7	0.16484
8	0.5389
9	0.18386
10	0.1286

Figure 2.4

Incubation time (hrs)	Lipase activity (U/ml)
1	0.10778
2	0.3742
3	0.62766
4	0.5426
5	0.4213
7	0.21132
9	0.10456
11	0.123456
13	0.01902
15	0.01268
17	0.03804
20	0.022345
22	0.0123

Figure 2.5 a

Carbon source (%)	Lipase activity (U/ml)
Soybean oil	0.999
Olive oil	0.8654
Coconut oil	0.6788

Figure 2.5 b

Soybean oil (%)	Lipase activity (U/ml)
2	0.10778
4	0.1585
6	0.4755
8	0.6657
10	1.02074
12	1.26166
14	0.8876
16	0.62766
18	0.4548
20	0.24726

Figure 2.6

Agitation Speed (rpm)	Lipase activity (U/ml)
50	0.99882
100	2.16194
150	1.0111
180	0.9756

Figure 2.7

pH	Relative activity (%)
Control	100
4	49
5	67
6	78
7	87
8	99
9	110
10	102
11	99
12	91

Figure 2.8

Temp (^{0}C)	Relative activity (%)								
Time(mins)	60	120	180	210	240	270	300	330	360
Control	100	100	100	100	100	100	100	100	100
30	98	90	87	72	71	67	60	52	47
40	103	98	100	74	70	64	60	54	40
50	88	75	67	64	58	54	45	38	40
60	80	73	66	65	60	53	49.9	38	36
70	73	68	60	56	50	47	42	36	30
80	67	61	53	43	40	32	30	27	21
90	62	56	44	39	29	21	18	15	12

Figure 2.11

Metal ions (1mM)	Relative activity (%)
Control	100
KCl	62.55
$HgCl_2$	100
NaCl	37.5
$MgCl_2$	75
$CoCl_2$	62.5
$FeCl_2$	100
$CaCl_2$	175
$MnCl_2$	150

Figure 2.9

NaCl (%)	Relative activity (%)					
Time(hrs)	1	3	6	9	12	24
Control	100	100	100	100	100	100
5	97	91	89	83	81	70
10	92	89	82	79	73	68
15	90	71	69	60	55	40
20	83	62	56	42	30	29
25	73	60	52	40	35	51
30	42	31	27	18	9	5

Figure 2.13

Vitamin (1mM)	Relative activity(%)
Control	100
Thiamine	172
Pyridoxine	63.5
Vitamin C	155
Nicotinic acid	187

Figure 2.10

Organic solvents (%)	Relative activity (%)			
	10%	20%	30%	40%
Control	100	100	100	100
Benzene	143	125	102	91
Xylene	168	157	132	83
n-hexane	100	98	89	72
Toulene	141	132	104	87
Butanol	87	95	90	63
Methanol	74	85	83	62
Ethanol	100	102	104	85
n-octane	105	100	100	66

Figure 2.12

Surfactants (1mM)	Relative activity (%)
Control	100
Tween 40	118.2
Tween 80	109
Triton X-100	100

	Chloroform	106 103 105 59	
	Figure 2.14	**Figure 2.15**	
	Chelators (1mM) **Relative activity (%)**	**1/S** **1/V**	
	Control 100	2 5.1 1.3 4.65 1 3.6 0.66 2.7	
	o-phenanthroline 87.5	0.5 1.99 0.4 1.22	
	EDTA 150	0.3 0.99 0.28 0.87 0.25 0.86 0.22 0.85 0.2 0.85	

Appendix B

Figure 3.2(a)		Figure 3.2 (b)		Figure 3.2 (c)	
Pm	**Fitness value**	**Pop. size**	**Fitness value**	**No. of generations**	**Fitness value**
0.0001	0.158181	10	0.159269	25	0.157084
0.0003	0.157433	20	0.161007	40	0.156271
0.0005	0.157505	30	0.158101	46	0.15552
0.0007	0.160521	40	0.161793	50	0.15552
0.0009	0.157141	50	0.158467	75	0.15552
0.0011	0.160521	60	0.157831	93	0.155471
0.0013	0.158102	70	0.16099	94	0.155406
0.0015	0.015685	80	0.156857	97	0.155388
0.0017	0.158546	90	0.157669	100	0.155388
0.0019	0.158578	100	0.156226	125	0.155388
0.0021	0.158383	110	0.158698	132	0.155361
0.0023	0.157887	120	0.158919	150	0.155361
0.0025	0.157778	130	0.15626	156	0.155345
0.0029	0.157203	140	0.157333	160	0.155318
0.0031	0.162118	150	0.159564	169	0.155303
		160	0.156351	172	0.15489
		170	0.156739	175	0.15489
		180	0.156949	200	0.154
		190	0.156471	225	0.15489
		200	0.156102	250	0.15489
		210	0.15552	275	0.15489
		220	0.156132	300	0.15489
		230	0.155603	350	0.15489
		240	0.156151	375	0.15489
		250	0.156183	400	0.15489
		260	0.155589	425	0.15489
		270	0.155964	450	0.15489
		280	0.156635	475	0.15489
		290	0.155602	500	0.15489
		300	0.156761	600	0.15489
		310	0.156439	700	0.15489
		320	0.157073	800	0.15489
		330	0.029412	815	0.15489
		340	0.156125	900	0.154886
		350	0.155728	1000	0.154886

Appendix C

Figure 4.1

Detergents (7mg/ml)	Relative activity (%)
Control	100
Surf excel	92
Tide	90
Wheel	109
Ariel	102
Nirma	104
SDS	106
Tween80	106

Figure 4.2

Oxidizing agents (%)	Relative activity (%)			
	Control	Hyrdogen Peroxidise	Sodium Perborate	Sodium hypochlorite
0.50	100	98	95	95
1.00	100	95	89	87
1.50	100	92	82	76
2.00	100	90	76	65

Figure 4.3

Detergent conc. (%)	Oil removal (%)	
	B+D	B+D+L
0.3	25	51
0.5	44	57
0.7	41	48
0.9	32	36

Figure 4.4

Lipase units (U/ml)	Oil removal (%)	
	B+L	B+D+L
20	20	42
40	26	55
60	25.8	54.7
80	25.4	54.5
100	25.7	54.3

Figure 4.5

Washing temp. (°C)	Oil removal (%)			
	B	L+B	D+B	B+D+L
34	10	15	30	44
37	16	29	38	52
40	18	26	45	60
43	15	22	37	51
45	12	19	31	48

Figure 4.6

Washing time (mins)	Oil removal (%)			
	B	L+B	D+B	B+D+L
15	12	24	35	52
30	20	36	38	59
45	18	38	41	62
60	14	31	37	56
75	10	25	33	51

Appendix D

Figure 5.2(a)		Figure 5.2(b)		Figure 5.2 (c)	
Pm	**Oil removal (%)**	**Pop size**	**Oil Removal (%)**	**Generations**	**Oil Removal (%)**
0.0001	0.01260148	10	0.01280877	5	0.01283772
0.0003	0.126822	20	0.01272184	8	0.0127451
0.0005	0.1266875	30	0.01278362	25	0.0127451
0.0007	0.1269314	40	0.01260364	36	0.01274128
0.0009	0.01269539	50	0.01265225	39	0.0126815
0.0011	0.01263029	60	0.01265844	46	0.01264669
0.0013	0.01273805	70	0.01263983	48	0.01258952
0.0015	0.01262267	80	0.01260148	75	0.01258952
0.0017	0.012138	90	0.01262861	108	0.01258603
0.0019	0.01269539	100	0.01258952	126	0.0125727
0.0021	0.01261087	110	0.0126181	150	0.0125727
0.0023	0.01269912	120	0.01273773	184	0.01257118
0.0025	0.01269912	130	0.01263859	192	0.01256676
0.0029	0.01273806	140	0.01258982	228	0.01256295
0.0031	0.01260582	150	0.01263258	250	0.01256295
0.0033	0.0127361	160	0.01262758	275	0.01256295
0.0035	0.01269715	170	0.01268443	300	0.01256295
0.0037	0.01266667	180	0.0126213	325	0.01256295
0.0039	0.01269894	190	0.0126093	350	0.01256295
0.0041	0.01273923	200	0.01261889	380	0.01256115
		210	0.01267746	406	0.01255853
		220	0.01267746	432	0.01255719
		230	0.01259034	450	0.01255719
		240	0.01264863	475	0.01255719
		250	0.01265193	500	0.01255719
		260	0.01260963	525	0.01255719
		270	0.01262897	550	0.01255719
		280	0.01264019	575	0.01255719
		290	0.01261513	600	0.01255719
		300	0.01259983	625	0.01255719
		310	0.01262246	650	0.01255719
		320	0.01262158	675	0.01255719
		330	0.01263699	700	0.01255719
				725	0.01255719
				750	0.01255719
				725	0.01255719
				750	0.01255719
				775	0.01255719
				800	0.01255719
				825	0.01255719
				850	0.01255719
				875	0.01255719
				900	0.01255719
				925	0.01255719
				950	0.01255719
				978	0.0125569
				1000	0.0125569

		1050 1100 1150 1200	0.0125569 0.0125569 0.0125569 0.0125569

Appendix E

Figure 6.1			Figure 6.2		
Fraction No.	**O.D at 280nm**	**Specific activity (U/mg)**	**Fraction No.**	**O.D at 280nm**	**Specific activity(U/mg)**
0	0	0	0	0	0
3	0	0	3	0	0
5	0	0	5	0	0
7	0	0	7	0	0
9	0	0	9	0	0
11	0	0	11	0	0
13	0	0	13	0	0
15	0	0	15	0	0
17	0	0	17	0	0
18	0	0	40	0	0
19	0	0	19	0	0
21	0	0	21	0.02	0
23	0	0	23	0	0
25	0	0	25	0	0
27	0	0	27	0	0.5
29	0	0	29	0	0.8
30	0	0	30	0	0.45
33	0	0	33	0	0
35	0	0	35	0	0
37	0	0	37	0	0
39	0	0	39	0	0
41	0	0	41	0	0
43	0	0	43	0	0
45	0	0	45	0	0
47	0	0	47	0	0
51	0	0	51	0	0
53	7	7	53	0	0
55	11	11	55	0	0
57	17.6	17.6	57	0	0
59	14	14	59	0	0
61	10	10	61	0.1	12
63	8	8	63	0.55	32.5
65	7.5	7.5	65	0.06	8
67	7	7	67	0.01	3
69	6	6	69	0	0
71	5	5	71	0	0
73	0	0	73	0	0

Appendix F

C program used for binary coded GA optimization for lipase production & biodetergent formulation:

```
#include "external.h"
#include <math.h>
int pow_of_2(),mask=1;
static int field_size[5];
static double XMAX[5],XMIN[5];
double wt[5];
static int no_var;
application() /*This routine should contain any application-dependent
              computations that should be performed before each  GA
                    cycle called by main().                    */
{
}
 app_data() /* Application dependent data input, called by init_data()
            Ask your input questions here, and put output in global
               variables.   In this  application, the utility  pointer
               of each individual is assigned a vector of integer that
               that will be used to store the interpreted chromosome*/
{
    int i;
     if(numfiles==0)    {
     //fprintf(outfp,"Enter the no of variables\n");
     //fscanf(infp,"%d",&no_var);
      no_var = 5;
           }
    for(i=0; i<no_var; ++i)
     {
       if(numfiles == 0) {
      /* fprintf(outfp,"Enter field size of variable no.%d    ", i+1);
       fscanf(infp,"%d",&field_size[i]);
           fprintf(outfp,"upper bound of variable no %d      ", i+1);
           fscanf(infp,"%lf", &XMAX[i]);
           fprintf(outfp,"lower bound of variable no %d       ", i+1);
           fscanf(infp,"%lf", &XMIN[i]);*/
           field_size[i]= 10;
           XMAX[0]=30.0; XMIN[0]=40.0;
           XMAX[1]=10.0; XMIN[1]=14.0;
           XMAX[2]=8.0; XMIN[2]=12.0;
           XMAX[3]=7.0; XMIN[3]=9.0;
      XMAX[4]=2.0; XMIN[4]=4.0;
           }
           }
}
 app_free()        /* This routine should free any memory allocated
```

```
                    in the application-dependent routines, called
                            by freeall() . */
{
    int i;
   for(i = 0; i < popsize; i++)
    {
    free(newpop[i].utility);
    free(oldpop[i].utility);
    }
}
 app_init()         /*  Application dependent initialization routine
                            called by initialize(). */
{
}
 app_initreport()   /* Application-dependent initial report called by
                            initialize() */
{
}
 app_malloc()       /* Application dependent malloc() calls, called by
                            initmalloc() */
{
    unsigned nbytes;
    int i;
     nbytes = no_var* sizeof(int);
    for(i = 0; i < popsize; i++)
    {
       if((newpop[i].utility = (int *) malloc(nbytes)) == NULL)
          nomemory(stderr,"newpop utility");
       if((oldpop[i].utility = (int *) malloc(nbytes)) == NULL)
          nomemory(stderr,"oldpop utility");
    }
}
 app_report()    /* Application-dependent report, called by report()*/
{
}
app_stats(pop)      /* Application-dependent statistics calculations
                            called by statistic() */
struct individual *pop;
{
}
 objfunc(critter)    /* Application dependent objective function */
struct individual *critter;
{
int i,n,p,start,stop,count,decoded,decoded1,neuron;
int j,k,q,start1,stop1,bitpos,bitvalue[50];
double fitness;
unsigned mask=1,tp;
float totdistance();
```

```
critter->fitness= 0.0;
            start=stop=0.0;
             for(k=0;k<chromsize;k++)   {
             if(k==(chromsize-1)) stop=lchrom-(k*UINTSIZE);
             else stop=UINTSIZE;
             tp=critter->chrom[k];
            // printf("\nstop=%d",stop);
             for(j=1; j<=stop; j++)  {
             bitpos = j + UINTSIZE*k;
             if((tp&mask)==1) { bitvalue[bitpos]=1;}
             else { bitvalue[bitpos]=0;}
            //printf("\nbitvalue[%d]=%d",bitpos,bitvalue[bitpos]);
             tp=tp>>1;
             }
             }
            //writechrom(critter->chrom);
            start=stop=0.0;
            for(i = 0; i < no_var; i++) {
            /* section of chromosome containing current integer field */
       start = stop + 1;
            stop  = start + field_size[i] - 1;
            //printf("\nstart=%d stop=%d", start,stop);
            //if(stop>(lchrom-20)) stop=lchrom-20;
            decoded=0;
            for(k=start; k<=stop; k++)  {
            count = stop-k;
            decoded+=bitvalue[k]*pow_of_2(count);
            //printf("\n count=%d",count);
             }
      /* convert bit field in chromosome to an integer and store it decoded */
       wt[i] = ((decoded * (XMAX[i]-XMIN[i]))/(double)(pow_of_2(field_size[i]) - 1.0)) +
XMIN[i];
//          printf("\n dd=%lf",wt[i]);
            }
            //printf("\n---Hi\n");
   critter->fitness=totdistance(wt);
//printf("fitness value=%f\n",critter->fitness);
  }
 int pow_of_2(j)
  int j;
  {
   int i,p;
   p=1;
   if(j==0)
      return(p);
   for(i=0; i<j; i++)
      p  *=2;
      return(p);
```

```
   }
  /*getarray(sarray,size)
    int *sarray,size;
    {
          int i,*temparray,temp,pos;
          temparray=(int *)malloc(popsize * sizeof(int));
          for(i=0; i<popsize; i++) temparray[i]=i;
          for(i=0; i<popsize-1; i++)   {
               pos=rnd(i,popsize);
               temp=temparray[pos];
               temparray[pos]=temparray[i];
               temparray[i]=temp;
               }
      for(i=0; i<size; i++)  sarray[i]=temparray[i];
      free(temparray);
      }*/
/*phenoshare(pop)
phenotypic sharing
struct individual *pop;
{
   int i,j;
   double dvalue,d,nichecount=0.0,pow();
   int *sarray;
 for (j=0; j<popsize; j++) {
       if (dshare == 0.0) nichecount = 1.0;
          else {
            dvalue = decodedvalue(pop[j]->chrom);
            if (sampleshare == 1) {
                    sarray = (int *)malloc(sampleshrsize * sizeof(int));
                    getarray(sarray,sampleshrsize);
                    for (i=0; i<sampleshrsize; i++) {
                       d = abs(dvalue - decodedvalue(pop[sarray[i]]->chrom));
                       if (d < dshare)
                           nichecount += pow(1.0-d/dshare,alpha);
                    }
                    free(sarray);
            } else {
                    for (i=0; i<popsize; i++) {
                       d = abs(dvalue - decodedvalue(pop[i]->chrom));
                       if (d < dshare)
                              nichecount += pow(1.0-d/dshare,alpha);
                   }
            }
          }
          pop[j].fitness /= nichecount;
   }
} */
```

List of publications and Conference papers

List of Publications in peer-reviewed international journals

1) Chauhan M, Srivastava M and Garlapati VK (2014) Purification, homology modelling and docking studies of an extremophilic lipase from *Staphylococcus arleatte* JPBW-1. Journal of Molecular Modelling and Graphics (Under Review).
2) Chauhan M and Garlapati VK (2014) Modelling Embedded Optimization Strategy for Formulation of Bacterial Lipase based Bio-detergent. Industrial & Engineering Chemistry Research 53(2):514-520. (**Impact Factor**: **2.206**)
3) Chauhan M, Chauhan RS, Garlapati VK (2013) Evaluation of a new lipase from *Staphylococcus sp*. for detergent additive capability. BioMed Research International, vol. 2013, Article ID 374967, 6 pages, 2013. (**Impact Factor**: **2.880**)
4) Chauhan M and Garlapati VK (2013) Production and characterization of a halo-, solvent- thermo tolerant alkaline lipase by *Staphylococcus arlettae* JPBW-1, isolated from rock salt mine. Applied Biochemistry and Biotechnology 171:1429-1443. (**Impact Factor**: **1.893**)
5) Chauhan M, Chauhan RS, Garlapati VK (2013) A novel extremophilic lipase production by *Staphyloccus arleatte:* Modeling and optimization through statistical integrated artificial intelligence approach. Enzyme Research, vol. 2013, Article ID 353954, 8 pages, 2013. (**Awaiting Impact Factor**)

Conference papers

1. Mamta Chauhan and VK Garlapati. Production of solvent, detergent and thermo-halotolerant lipase by *Staphylococcus arlettae* in submerged fermentation. International Conference on Industrial Biotechnology held in Punjabi University, Patiala Punjab, India from November 21-23, 2012.
2. Mamta Chauhan and VK Garlapati. A novel thermoactive and alkaline lipase from Staphylococcus arlettae as an addictive in detergent formulations: a statistical approach. International Conference on Advances in Biotechnology and Bioinformatics held at Dr. Y.P. Patil Biotechnology and Bioinformatics Institute, Pune 26-28 Nov. 2013