Optimization Study Of Lipase From Lactic Acid Bacteria And Synthesis Of Flavor Esters

THESIS

Submitted in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

By

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2015

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CERTIFICATE

This is to certify that the thesis entitled **"Optimization Study Of Lipase From Lactic Acid Bacteria And Synthesis Of Flavor Esters"** was submitted by Sita Ramyasri Uppada, ID. No. 2010PHXF803H for the award of Ph. D. degree of the Institute embodies original work done by her under my supervision.

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ACKNOWLEDGEMENT

First of all, I would like to thank the Almighty for giving me the opportunity to do this research work with good health and prosperity.

My heart-felt gratitude to ***Prof. V.S.Rao***, Director (BITS-Pilani, Hyderabad Campus), for permitting me to carry out my research work in the Biological Sciences Department.

I wish to express my sincere gratitude and heartfelt thanks to my guide ***Dr. Jayati Ray Dutta***, Department of Biological sciences, for her excellent guidance, constant encouragement, enormous patience and good advice with a smiling face throughout my research work. I would like to acknowledge her for her moral support at every instance, without which, this work would not have been completed. I owe it to you mam.

I am grateful to ***Prof. Suman Kapur***, Head of Department of Biological Sciences for giving me an opportunity to carry out my research work in the laboratory.

I would like to extend my gratitude to my Doctoral Advisory committee members, ***Dr. Anupam Bhattacharya,*** Department of Chemistry and ***Dr. Palash Mandal,*** Department of Biological Sciences, for their valuable suggestions.

I would like to express my heart-felt gratitude and thanks to ***Prof. Vidya Rajesh***, Dean, Academic Research Division, for providing me with fellowship and contingency to carry out my research work.

I wish to express my thanks and take immense pleasure in acknowledging all the faculty of Department of Biological Sciences, BITS, Pilani-Hyderabad Campus for their support and encouragement to carry out this research work.

I express my thanks to laboratory technicians, Department of Biological Sciences for all their help in one way or the other.

Lastly, but most importantly I would like to acknowledge my parents, U. Pradeep kumar and U. Aruna Kumari, brother, parent-in-laws K. Bhaskara Rao and K. Nagamani and brotherinlaw for their amazing support and my reason to smile even on difficult days. I heartfully acknowledge my husband K. Leela Ravi Teja, for his support, I would never have been able to achieve this dream without his constant encouragement. My thesis will not be complete without my daughter Jasmitha Lasya's presence.

Abstract

Lipases play a very important role as natural biocatalysts catalyzing several biochemical reactions. There has been an increased demand for microbial lipases due to inability of plant and animal lipases to meet the global requirement. The current focus of the study was intended to isolate hyperactive lipase producing strains using low cost substrates. The physical parameters for production of lipase from the selected strains were optimized by both classical and statistical methods, followed by cloning and expression to further improve the yield. The structure-function relation can be well understood by cloning and sequencing which helps in search for different sources of lipase for various biotechnological applications. Finally the expressed lipase was immobilized for esterification reactions for producing short chain fatty acids which can be used as flavor agents in food industries.

In the present study, the best possible combinations of lipase producing Lactic acid bacteria were selected. Different single cultures like *Lactococcus lactis*, *Lactobacillus brevis*, *Lactobacillus plantarum* and co-cultures like *Lactococcus lactis* + *Lactobacillus brevis*, *Lactococcus lactis* + *Lactobacillus plantarum* and *Lactobacillus brevis* + *Lactobacillus plantarum* were studied for the optimum production of lipase using olive oil as substrate.

The optimum conditions for maximum activity of different lactic acid bacterial lipase were investigated. The lipase activity with classical method of optimization was 32 U/ml from co-culture of *Lactococcus lactis* + *Lactobacillus brevis*, 35 U/ml for *Lactococcus lactis* + *Lactobacillus plantarum* and 37 U/ml for *Lactobacillus brevis* + *Lactobacillus plantarum* whereas with the statistical optimization the activity was found to be 33.5 U/ml from co-culture of *Lactococcus lactis* + *Lactobacillus brevis*, 36 U/ml from *Lactococcus lactis* + *Lactobacillus planatrum* and 38.5 U/ml for *Lactobacillus brevis* + *Lactobacillus plantarum* which showed 2

fold increase in the lipase production. The statistical approach was more accurate in the production of lipase from both single and co-cultures of different lactic acid bacteria when compared to the classical OFAT (one factor at a time) method of optimization.

Mutagenic studies with the parent strains were done by UV, heat and ethidium bromide. A few colonies were screened from the selected media for lipase study. Amongst all, the best mutant was isolated after 120 minutes of UV treatment where there was increase in lipase activity. The three mutant co-culture combinations showed increase in lipase activity of 34 U/ml for *Lactococcus lactis* + *Lactobacillus brevis,* 35 U/ml for *Lactococcus lactis* + *Lactobacillus plantarum* and 39 U/ml for *Lactobacillus brevis* + *Lactobacillus plantarum.* The mutant was found to be sensitive to penicillin at higher concentration of 400 µg and resistant to ampicillin and streptomycin at all concentrations and a hyper producer of protease in addition to lipase.

Heterologous expression system was developed for improving the strain for increased lipase production. The lipase gene from *Lactobacillus brevis* and *Lactobacillus plantarum* were cloned and expressed in *E. coli* BL21 (DE3) pLysS with vector pMALc5X. The optimum pH and temperature for lipase obtained after studying the kinetic parameters was found to be pH 7 and 37 ºC for *Lactobacillus brevis* and pH 6 and 40 ºC for *Lactobacillus plantarum.* The V_{max} and K_m values of the lipase from *Lactobacillus brevis* were 1.28 µmol/mg/min and 0.44 mM and for *Lactobacillus plantarum* were 1.44 µmol/mg/min and 0.426 mM respectively. The expressed lipase of *Lactobacillus brevis* showed a 3 fold increase in specific activity (36.42 U/mg) after expression than that of native protein (12.5 U/mg) before expression. Similarly the expressed lipase of *Lactobacillus plantarum* showed a 3.3 fold increase in specific activity (42.8 U/mg) after expression than that of native protein (13.2 U/mg).

The lipase produced was then immobilized using sodium alginate. The immobilized lipase showed optimal conditions of pH 7 and 40 °C for *Lactobacillus brevis* and pH 6.5 and 45 °C for *Lactobacillus plantarum.* The V_{max} and K_m values of the immobilized enzyme for *Lactobacillus brevis* were 1.33 µmol/mg/min and 0.39 mM and 1.47 µmol/mg/min and 0.37 mM for *Lactobacillus plantarum.*

The application of immobilized lipase from *L. plantarum* and *L. brevis* was then studied for various industries. Degradation of fat is an important property of lipases. In the present study degradation of fat was observed at 96 hrs for *L. brevis* and 72 hrs for *L. plantarum*. The recombinant lipase showed complete degradation in 48 hrs. Similarly lipase mediated esterification was studied using different substrates. The effect of parameters for esterification reaction like suitable solvent, reaction time, temperature, agitation, substrate and enzyme concentration was optimized. Different esters like 2, 3 and 4-hydroxybenzyl acetates and triazole esters were synthesized which makes a great impingement in natural flavor industry by introducing products synthesized by immobilized lipase from *L. plantarum*.

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Abbreviations

α	Alpha
ANN	Artificial Neural Network
β	Beta
DNA	Deoxyribonucleic acid
EtBr	Ethidium Bromide
GA	Genetic algorithm
IR	Infrared spectroscopy
I	Inoculum
I.P	Inoculum volume
Lc	*Lactococcus lactis*
LBb	*Lactobacillus brevis*
LBp	*Lactobacillus plantarum*
lt	Litre
µmol	Micromoles
Min	Minute
mg	Milligrams
ml	Millilitre
mM	Millimolar
NTG	Nitrosoguanidine
NMR	Nuclear Magnetic Resonance spectroscopy
RSM	Response Surface Methodology
S	Substrate

SVM	Surface Vector Machine
sp	Species
TLC	Thin Layer Chromatography
Tris-Cl	Tris-chloride
Temp	Temperature
U	Units
UV	Ultraviolet
w/v	Weight/Volume

INTRODUCTION

1.1 Lipases

Chemical catalysts like acids and bases require high temperature, pressure, acidic or alkaline pH leading to higher capital investment, special equipment and control systems [1]. Unwanted by-products and difficulty in product recovery also add to the main drawbacks of chemical catalysts. All these demerits have turned the research focus onto selectively specific, biodegradable, easy-to-recover, and reusable bio-molecules called enzymes, making them potential surrogates for chemical catalysts. These biological catalysts include both extra-cellular and intracellular enzymes [2]. One such important bio-catalyst of industrial importance is the enzyme lipase.

Claude Bernard first discovered lipases in 1856 while studying the role of the pancreas in fat digestion [3]. Lipases belong to the enzyme class of hydrolases which hydrolyse triglycerols to free fatty acids and glycerol [4]. They are also capable of governing different reactions like esterification, trans-esterification, hydrolysis, acidolysis and aminolysis [5].

Hydrolases consists of two major classes like esterases and lipases. Esterases (EC 3.1.1.1) hydrolyze esters and usually only triglycerides composed by short chain fatty acids and lipases (EC 3.1.1.3) are active against water-insoluble substrates and hydrolyze long chain fatty acids. The property of interfacial inactivation was observed only with lipases as shown in Figure 1.1. The commercial applications of lipases is due to their multifold properties, easy extraction procedure, very less temperature and pressure conditions and potential of unlimited supply which is lacking in traditional industrial processes.

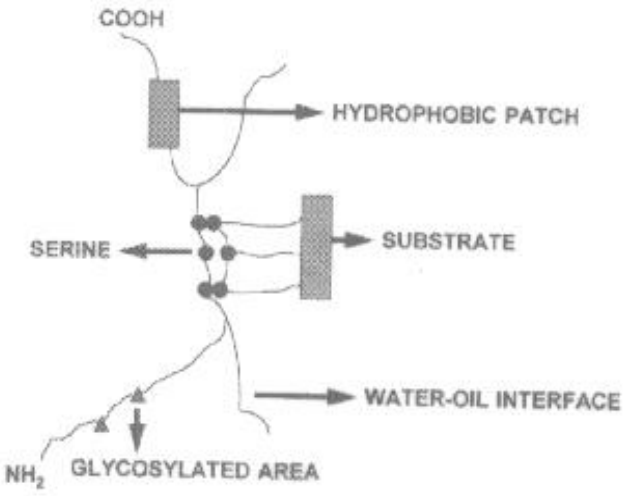


Figure 1.1: Lipase

(Source: Jaeger, et al. 1999)

1.2 Sources of lipase

Lipases are derived from various sources like plants, animals, human, bacteria, yeast and fungi [6]. In plants, oil seeds, cereals and laticifers are the main lipase reserves. Due to high triglycerol content, lipases can also widely be derived from corn, sunflower, rapeseed, castor and sesame seeds [7-9]. The function of these enzymes in plants is not fully elucidated but is known be involved in some metabolic reactions and defense against external agents. Similarly animals like porcine, pigs, goat, sheep and calves are good source of lipases [10].

Although lipases are obtained from different sources, microbial enzymes derived from bacteria, yeast and fungi are often used for their high catalytic activity, rapid microbial growth, ability of microorganism to withstand high pH and temperature, easy cultivation with cheap substrates, easy to manipulate genetically and more product formation. Microbial lipases are more stable than plant and animal derivatives [11]. The best studied lipase producing bacteria like *Serattia, Pseudomonas aeruginosa* and *Pseudomonas fluorescens* were the first organisms to be isolated and studied for presence of lipase in 1901 [12]. The presence of lipases was also seen in few bacterial, fungal and yeast sources that are listed below in Table 1.1.

Table 1.1: The occurrence of lipases in different microorganisms

Organism	Author name and year	Reference number
Pseudomonas cepacia	Sugihara et al., 1992	13
Pseudomonas aeroginosa	Kukeraja et al., 2005	14
Staphylococcus sp	Ajith kumar et al., 2012	15
Achromobacter lipolyticum	Khan et al., 1967	16
Bacillus cereus	Ananth et al., 2013	17
Bacillus licheniformis VSG1	Sangeetha et al., 2010	18
Bacillus smithii BTMS 11	Ephraim et al., 2014	19
Bacillus megaterium AKG-1	Sekhon et al., 2006	20
Bacillus stearothermophilus MTCC 37	Saba et al., 2012	21
Bacillus pumilus RK-31	Kumar et al., 2012	22
Candida Antarctica ZJB09193	Liu et al., 2012	23
Candida rugosa	Pereira et al., 2001	24
Lactobacillus brevis	Chander et al., 1973	25
Lactococcus lactis	Uppada et al., 2012	26
Lactobacillus plantarum	Lopes et al., 1999	27
Enterococcus faecium MTCC 5695 *Pediococcus acidilactici*MTCC 11361	Rama Krishnan et al., 2013	28
Kluyveromyces marxianus	Deive et al., 2003	29
Lactobacillus delbrueckii subsp. bulgaricus	El-Sawah et al., 1995	30
Lactococcus heleveticus	Rashmi et al., 2014	31
Aspergillus niger	Faloni et al., 2006	32
Aspergillus awamori	Basheer et al., 2012	33
Aspergillus fumigatus MTCC 9657	Rajan et al., 2011	34
Fusarium solani FS-1	Maia et al., 1999	35
Bacidiobolus	Okafor et al., 1990	36
Thermomyces lanuginosus	Li et al., 2009	37

Thus most of the bacterial and fungal species are studied for lipase production.

1.3 Structure of lipase

The lipase contains α/β hydrolase fold which includes a central, eight- stranded β sheet surrounded by α helices [38] on both the sides as shown in Figure 1.2. The number of β sheets will differ from each organism. Each α/β hydrolase enzyme contains catalytic triad containing nucleophilic residues Serine (Ser), Histidine (His) and Aspartate (Asp)/ Glutamate (Glu), where Serine is the nucleophile, Histidine is the basic residue, and aspartate or glutamate is the acidic residue. Serine residueis located in a highly conserved Gly-X-Ser-X-Ser pentapeptide. The pentapeptide also differs from organism to organism, with alanine substituting glycine in some microbes. Lipases attain a lid like structure which contains one or two α helices. This property of lipase leads to its conformational change when in an interface between oil and water [39] (called interfacial activation phenomenon). Open or closed position of the lid determines the active or inactive conformation of the enzyme, respectively [40]. The open form of the enzyme gives the binding accessibility to the substrate.

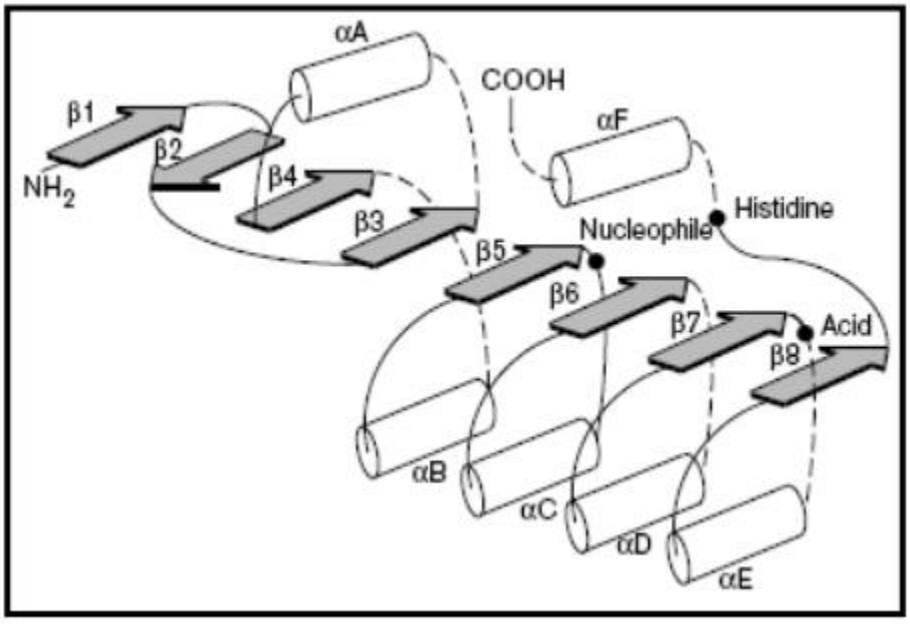


Figure 1.2: showing the lipase structure (α/β hydrolase)

(Source: Jaeger, et al. 1999)

1.4 Application of lipases

1.4.1 Lipases in detergent industry

The first commercial lipase used in detergentswas isolated from *Thermomyces lanuginosus* [41]. Lipase isolated from *Staphylococcus arlettae* JPBW-1 of rock salt mines, showed an ability to remove oil stains. Hence, it was reported as an additive in laundry detergent formulations [42]. Alkaline lipase from *Staphylococcus* sp. strain ESW showed extreme stability against non-ionic and anionic surfactants thereby highlighting its usage in detergent formulations [43]. An alkaline lipase isolated from rubber seed was studied for its compatibility with other commercial detergents and had been found to be functional in alkaline pH and low temperature. It was also resistant towards various surfactants and detergents which in turn was used as an additive in the detergent formulations [44].

1.4.2 Lipase in production of biodiesel

The potential use of lipases in the production of biodiesel was discovered in a comparative study done on *Candida antarctica* (Novozym 435) and *Photobacterium lipolyticum* lipase (M37) [45]. The results showed M37 to be the best suitable strain for biodiesel production. Also a noble biofuel, 'Ecodiesel-100' produced by pig pancreatic lipase, showed biochemical properties similar to that of the conventional biodiesel [46]. Methyl ester, a promising catalyst in biodiesel production was obtained in high yields by *Proteus mirabilis* [47]. A novel lipase CS274, isolated and purified from *Ralstonia sp*. showed resistance against both the oxidizing and reducing agents and it is used in the production of biodiesel from palm oil [48].

1.4.3 Lipases in ester synthesis

Short chain esters have great significance in enhancing flavors and hence are used in food industries. Methyl ester of pine apple flavor and octyl acetate of orange flavor were

synthesized by immobilized *Rhizopus oryzae* NRRL 3562 lipase which was used in food and beverage industries [49].

Lipase isolated from marine actinomycete, *S. variabilis* NGP 3 produced a fragrant ester which is potentially used in textile and brewing industries [50]. Ethyl valerate, a green apple flavor ester, is produced by esterification of valeric acid with ethyl alcohol by immobilized *Burkhoderia cepacia* [51].

Different esterification and transesterification reactions have been performed by lipase derived from *Rhizopus* sp. to produce citronellyl esters like citronellyl acetate and citronellyl butyrates which are used as flavor and fragrance enhancers respectively [52].

1.4.4 Lipase as biosensors

Potentiometric biosensors developed by lipase of *Candida rugosa* are used in the detection of organophosphorus pesticides like methyl-parathion and tributyrin in agriculture [53]. Fungal derived lipase-labelled probes are used in the detection of the complementary nucleic acids by specific hybridization which potentially surrogates the highly unstable and hazardous radiolabelled polynucleotides [54]. Lipases also show their proficiency in bio-sensing the blood cholesterol and triglycerides [55].

1.4.5 Lipase in cosmetics

Lipases derived from *Candida cylindracea*, *Rhizomucor meihei*, *Candida antarctica*, areused in self-care products such as hair weaving preparations and anti-obesity creams [56].

1.4.6 Lipases in medical treatment

Lipases obtained from *Yarrowia lipolytica* are used in the treatment of exocrine pancreatic insufficiency [57]. Lipases in combination with hyaluronidase enzyme are effectively used in the treatment of skin inflammations [58]. Lovastatin, a drug synthesized by lipase derived from *Candida rugosa* is prescribed for the control of serum cholesterol level [59]. Lipase isolated from *Serratia marcescens* shows its capability in the asymmetric

hydrolysis of 3-phenylglycidic acid ester which is a key intermediate in the synthesis of diltiazem hydrochloride [60].

1.4.7 Lipases in oil degradation

Candida antarctica, Pseudomonas cepacia, Yarrowia lipolytica, Pseudomonas aeruginosa and Carica papaya are known to be the potential sources for lipase that holds caliber in the degradation of oil spills, fats and grease from the oil contaminated water bodies thereby aiding in biological water cleanup, preventing water pollution [61].

Large success of microbial lipases in food and other biotechnological systems can be attributed to the broad biochemical diversity of the microorganisms, to the genetic manipulation of the organisms and to improved techniques for enzyme production and purification. Microbial enzymes also conform more closely to the required characteristics, such as cost of using the enzyme, activity at optimal conditions, safety of the enzyme and availability at required purity and stability, than do animal and plant lipases.

Commercial production of extra-cellular enzymes is in principle very simple, involving cultivation of a microorganism and subsequent recovery of the enzyme. This requires the use of an organism which grows on an inexpensive medium and produces high yields of enzyme in a short time. Simple and inexpensive recovery of the enzyme leading to a stable product with an acceptable appearance and which can be handled safely is also important.

These objectives are fulfilled by the combined optimization of strain properties and process parameters. Optimization of strain properties, mainly by the development of suitable mutants, usually offers an inexpensive and permanent solution to the problem.

1.5 Review of Literature

Lipases have wide industrial applications and therefore continuous efforts are being made by scientists to discover new hyperactive lipase producing organisms favoring cost-effective production and purification to make the process more attractive for commercial

exploitation. Different scientists have worked on lipase production by bacterial and fungal species and their work are reviewed under the following sub-headings**:**

1.5.1 Optimization of process parameters for lipase production by single and co-culture methodology

A lipase of 66 KDa was obtained from *Bacillus* sp. MPTK 912 after optimization of physico-chemical parameters and purification by sephadex-100 column [62]. From the study it was found that glucose and peptone acted as best carbon and nitrogen sources for increased lipase activity. There was a report that *Pseudomonas aeroginosa* isolated and screened from waste water has ability to produce lipase [63]. 6 isolates were examined for their lipase production. Maximum enzyme activity was achieved at pH 7 and 35 ^{o}C temperaturein presence of olive oil as substrate.

32 bacterial strains were isolated from soil samples of hot springs and screened for their lipase activity. The strain with highest activity was identified as *Geobacillus* sp [64]. Maximal enzyme enzyme activity was obtained with mustard oil as carbon source and yeast extract as sole nitrogen source at pH 8.5 and temperature 65 ^{o}C.The production of a lipase by *Bacillus licheniformis* sp. was optimized using olive oil and glucose as substrate. Production was maximum at 48 hrs with pH 8 and temperature 40 ^{o}C [65].

It is also reported that mesophilic bacteria like *Staphylococcus* sp. produce lipase with maximum enzyme activity of 13.92 U/ml at 40 ^{o}C and pH 7.0 [66]. After partial purification, the activity was observed to be 34.78 U/ml with 80% recovery of enzyme. Forty different microorganisms were studied for lipase production in which *Burkholderia cepacia* showed highest lipase activity with 0.0058 U/ml [67]. The effect of different parameters like pH, temperature, substrates for maximizing the lipase activity was studied for lipase produced from fish gut isolate *Vibrio* sp [68]. It was found that optimum pH 7 and temperature 37 $^{\circ}$C was required for maximum lipase production.

Staphylococcus sp. Lp12 isolated from oil contaminated soil was screened for lipase production and optimization for physical parameters was done. From optimization, it was found that peptone and starch acts as major sources of nitrogen and carbon sources. The optimum pH and temperature for maximum lipase production was investigated to be pH 8 and 45 ^{o}C [69]. Optimization of different parameters like pH, temperature, incubation time, carbon and nitrogen sources were optimized for lipase enzyme produced from *Serratia marcescens*. An optimum activity of 6.021 U/ml was obtained with pH 7 and 30 ^{o}C [70].

All the above reports showed production of lipase from single bacterial or fungal cultures. But microbial consortium consisting of two or more different microorganisms is largely responsible for several biotransformations in natural environment. Mixed culture fermentations are widely used in biotechnology for many processes including the production of antibiotics, enzymes, fermented foods, composting, dairy fermentation, bioconversion of apple distillery and domestic wastewater sludge. When compared to single cultures, co-cultures are highly advantageous because the synergistic utilization of metabolic pathways is more in co-culture.

Highest activity of 2.37 U/ml of cellulase was obtained by co-culture of *Aspergillus niger* and *Trichoderma viride* [71]. The co-culture of *Penicillium chrysogenum* and *Trichoderma viride* were grown for synthesis of tannase. The co-culture produced a highest activity of 84 U/g/min of tannase. The conditions were optimized and found that 96 hours of incubation was favorable for their highest yield [72].

Industrially important enzymes like cellulases and amylases were produced from co-culture of *Aspergillus oryzae* and *Trichoderma reesei*. The conditions for the production were optimized and found that pH 5.5 and temperature 30 °C were favorable for more production of enzymes. With the optimized conditions, an amylase activity of 0.787 IU and cellulase activity of 0.587 IU was obtained [73].

Lignolytic enzyme, laccases were obtained by co-culture of *Trametes maxima* and *Paecilomyces carneus*. The highest activity of 12,382.5 U/mg protein was obtained by optimized conditions using Plackett-Burman [74].

From the results of the above mentioned investigations it can be concluded that the production of enzyme by microbes can be changed if the parameters in the culture medium like carbon, nitrogen, and some physical parameters like pH, temperature etc. are varied and when compared to single cultures, co-cultures play key role in production of more enzymes.

1.5.2 Optimization of process parameters for lipase production using response surface, artificial neural network and genetic algorithm methods.

Conventional single factor optimization method requires number of experiments to determine the optimum levels and does not represent the combined effect of all the parameters involved. These limitations of single parameter optimization can be eliminated by optimizing all the affecting parameters collectively with statistical design techniques using Response Surface Method (RSM).

In recent years, this approach has become a very popular tool for optimization of various process parameters. But RSM too has some major drawbacks like, i) it is usually quadratic, thus being incapable to be applied in complicated nonlinear cases; ii) the co linearity problems between factors may exist; and iii) sensitivity analysis of input variables is difficult to perform because of the presence of cross interactions. These drawbacks of RSM can be removed by Artificial Neural Network (ANN).

An ANN is a highly interconnected network consisting of many simple processing elements capable of performing a massively parallel computation for data processing inspired by the elementary principles of the nervous system. An ANN imposes no restrictions on the type of relationship governing the dependence of the growth parameters on the various running

conditions [75]. ANN based simulators can also be used to generate response surfaces and has shown better predictability than other multivariate algorithm in many instances.

Development of high speed computing capability now paves the way for using complex and computationally intensive algorithms for on line process optimization. Among all the recent nonlinear optimization techniques genetic algorithm is the most studied and general one. Genetic algorithm (GA) from its inception found many promising and interesting applications in function approximation and optimization. Major advantage of genetic algorithm over other conventional optimization techniques is the flexibility it provides in framing the objective function and constraints. It can simultaneously handle multiple number of objective functions with linear, nonlinear equality and inequality constrains. These aspects along with ease of programming were major motivations behind its application for the present modeling.

The application of GA in bioprocess optimization has been reported in early 1996 [76-77] by many researchers. However, a comprehensive genetic optimization technique for predicting an optimum arbitrary feed-in profile has been reported [78]. A common feature of almost all the optimization techniques is to control feed-in, feed-out and recycling rate. But GA being so flexible tool for optimization, it is quite possible to seek for optimum values of other reaction parameters also, e.g., reaction time, amount of initial inoculum, initial substrate concentration etc. In this thesis emphasis has been given to see that these parameters are also playing important role to control the reaction dynamics.

In recent years, RSM was used to study the role of environmental factors affecting the growth of *Saccharomyces cerevisiae* [79]. The effect of microbiological parameters, viz., slant age, inoculum age and amount of inoculum were studied using RSM for production of pectolytic enzymes in submerged fermentation [80]. After optimization, the fermentation time was reduced from 144 to 120 hrs. RSM was applied for maximizing lipase production in

Bacillus subtilis. Maximum production of 16.627 U/min/ml was obtained with statistical optimization of culture media [81].

RSM has been used for optimization for the production of lipase from *Rhizopus delemar*. This method gave lipolytic activity of 1585 μmol/l min [82]. The effects of cultivation medium were optimized for the production of lipase from *Burkholderia* sp. HL-10 by RSM technique and found a threefold increase in activity. The optimized medium containing olive oil, tryptone and Tween-80 resulted in a maximum activity of 122.3 U/ml [83]. Different parameters were optimized for lipase mediated transesterification reaction from *Rhizopus oryzae* by RSM. Higher yield of biodiesel was obtained with pH 5.5, 150 rpm and olive oil as substrate [84].

RSM and ANN were applied for optimizing the physic-chemical parameters to enhance the production of lipase by *Bacillus sphaericus*. Maximum lipase activity of 4.45 U/ml was obtained with statistical optimization [85]. ANN has been applied to predict the enzymatic hydrolysis for the production of glucose which showed good results.

The culture conditions for production of alkaline lipase from *Staphylococcus arlettae* was optimized by RSM and GA. An optimized yield of 6.5 U/ml of enzyme was obtained and this extremophilic lipase could be applied in different industries [86]. ANN and GA were used to optimize the cultural conditions for production of actinomycin V from *Streptomyces triostinicus* [87]. A higher antibiotic yield was obtained by the ANN/GA which was 36.7% higher than the yield obtained with RSM.

Different cultural parameters such as incubation time, inoculum level, initial moisture content, carbon level and nitrogen level of lipase production from *Yarrowia lipolytica* NCIM 3589 [88] were optimized using Placket-Burman design and an activity of 18.58 U/mg was obtained by statistical optimization.

RSM and ANN both coupled with GA were used to optimize the biomass production from *Bifidobacterium bifidum* 255 and the optimal conditions for the maximal biomass yield obtained from statistical optimization were 37.4 ^{o}C, pH 7.09, inoculum volume 1.97 ml, inoculum age 58.58 h, carbon content 41.74% (w/v), and nitrogen content 46.23% (w/v) [89].

From the above reports, it is evident that RSM is a much better technique than single parameter optimization involving factorial design and regression analysis, which helps in evaluating the effective factors and building models to study the interaction and select optimum conditions of variables for a desirable response in microbial enzyme production. But ANN is superior and more accurate modeling technique when compared to RSM method as it represents the nonlinearities in a much better way. However, response surfaces plotted by 3-D plots can provide a good way for visualizing the parameter interaction. Therefore, now-a-days both techniques coupled with genetic algorithm were used in unison for predicting optimum fermentation process conditions for microbial enzyme production.

1.5.3 Optimization of process parameters for lipase production using Taguchi and Support Vector Machine method

Taguchi method facilitates the study of interaction of a large number of variables with less experimental trails. The method utilizes a fractional factorial design in form of an orthogonal array containing a representative set of all possible combinations of experimental conditions. The fermentation conditions for production of protease from *Bacillus subtilis* HB04 was optimized using Taguchi and found the contribution of five factors like carbon sources, nitrogen sources, metal ions, temperature and agitation on protease production [90]. Different parameters like medium composition, pH, temperature, mixing intensity and substrate particle size on lipase production from *Pseudomonas aeoroginosa* B-3556 was studied using Taguchi method. Maximum enzyme activity of 44.8 U/ml was obtained by optimization [91].

Parameters like carbon source, organic, inorganic nitrogen sources, agitation and metal ion for alkaline protease production from *Bacillus clausii* was studied using Taguchi method [92]. It was found that organic nitrogen and carbon sources play prominent role in the increased production. An increased production of 23% was obtained by optimizing the parameters for pectinase production from *Aspergillus niger* by Taguchi method [93].

Support Vector Machines (SVMs) is the most important method of supervised learning, which analyses and recognizes data patterns, useful for classification and regression. The parameters for production of maltooligosacchrides producing amylase from alkalophilic bacterium, *Streptomyces lonarensis* were optimized using SVR modeling [94]. A maximum activity of 297 U/ml was obtained by optimized operating conditions. SVM was applied to optimize optimal temperature for xylanase production from *Bacillus pumilus* [95].

The physical properties of soil like soil shear strength, soil aggregate stability were optimized using SVM. The results showed great accuracy in predicting the properties [96].

1.5.4 Strain Improvement by Mutagenesis

Strains can be improved by different methods like mutation, protoplast fusion or by using the recombinant DNA technology techniques like homologous and heterologous expression of desired gene. Mutation leading to discontinuous hereditary changes of genetic material can take place both spontaneously and by induction with mutagenic agents. In the literature much information is available on general methods and special techniques for induction and selection of mutants.

The most used agents in the mutation of microorganism are agents, such as N1-methyl – N1-nitro-N-nitrosoguanidine (NTG), Diethyl Sulfate (DES), Ethidium Bromide (EtBr) and Nitrous acid (HNO2) as well as gamma or ultra violet irradiation [97]. The introduction of mutation by N1- methyl-N1-nitro-N-nitrosoguadine (NTG) appears to be the closest system to in vivo ‘directed mutation’. NTG treatment causes cluster of mutation around the replicating

fork of the bacterial chromosome [98]. Among the other mutagenic agents the use of ultraviolet light is only one of the large numbers of physical agents which increase the mutation rate. The most effective wavelength of ultraviolet radiation for inducing mutation is about 2,600 angstrom. This is the wavelength that is best absorbed by DNA whereas protein absorbs a very little energy. When a substance absorbs sufficient energy from ultraviolet light, some of their electrons are raised to higher energy levels and the excited molecules becomes reactive and mutated. The ultraviolet radiation produces several effects on DNA. One such effect is the formation of chemical bonds between two adjacent pyrimidine molecules in a polynucleotide and particularly, between adjacent thymine residues.

As the two thymine residues associate or dimerize to form a dimer, their position in the double helix becomes so displaced that they can no longer form hydrogen bonds with the opposing purines and thus the regularity of the helix becomes distorted. The dimerization interferes with the proper base pairing of the thymine with adenine, and may result in thymines pairing with guanine [99]. UV, N-N-methyl-N-nitro-N-nitrosoguanidine and sodium azide were used as mutagenic agents for improving the strains like *Bacillus coagulans* and *Geobacillus sterothermophilus* for cellulase production [100]. From the results it was found that NTG has best mutagenic activity than other mutagenic agents.

The strain *Aspergillus flavus* was induced with UV mutations to enhance the production of alkaline protease and found that the activity increased with mutagenesis [101]. UV mutagenesis increased the production of lipase from *Acinetobacter calcoaceticus* D29 where 48.69% of more activity was observed than original strain [102]. Lipase producing *Sporobolomyces salmonicolor* OVS8 isolated from oil mill spillage was subjected to UV mutagenesis which showed 3.2 times increase in activity of lipase than parent strain [103]. Increased lipase production was observed with mutagenic strain of *Staphylococcus* sp which was subjected to random mutagenesis by UV [104]. *Serratia marcescens* ECU1010 was

subjected to UV mutagenesis [105] for strain improvement in order to enhance the lipase productionand showed increased activity of lipase and low cost of medium than compared to parent strain.

Cells of *Yarrowia lipolytica* CBS 6303 [106] were subjected to mutagenesis by N-methyl-N-nitro-N-nitrosoguanidine. The mutant strain showed an increase in activity of lipase from 28 U/ml to 1000 U/ml. Through their experiments it was also evident that the mutant showed similar properties as of wild strain. UV, Ethyl Methyl Sulphonate (EMS) and Ethidium bromide (EtBr) were used as mutagenic agents to enhance amylase production in *Aspergillus niger* and found that EMS-EtBr combination mutants showed 2-4 fold increase in activity of Glucoamylase [107].

From the above reports it was evident that strain improvement plays a key role in increasing the enzyme production at industrial level.

1.5.5 Heterologous expression and purification of lipase gene

Industrial scale production of enzyme will be feasible only when the desired protein is formed at high levels and the producing organism can be cultivated in a large-scale fermentation. Thus, the genes for many industrially important enzymes have been inserted in heterologous hosts such as bacteria, yeasts and fungi for elevating the expression levels. Hence the strains which were not improved for increased production of enzymes by mutations were subjected to heterologous expression. In heterologous gene expression, foreign gene was expressed by the host organism for increased production of desired products. In the literature much information is available on heterologous expression of desired gene.

lcc1 gene from *Trametes trogii* was isolated and expressed in the methylotrophic yeast *Pichia pastoris.* The expressed gene showed high productivity of laccases and high activity in presence of organic solvents [108]. Lipase gene from native strain of *Y. lipolytica* DSM 3286 and its mutant strain *Y. lipolytica* U6 were isolated [109] and cloned in *E. coli.*

Gene *lipZ03,* a novel lipase gene was isolated from soil and expressed in *Pichia pastoris*. From the above study it was found that the enzyme was highly stable in the temperature range of 40–60 °C alkaline pH 8–10. It also showed the capacity to hydrolyze methanol and produced biodiesel [110].

LipAMS8, a lipase gene from psychrophilic bacteria*Pseudomonas* AMS8 was isolated, sequenced and cloned in *E.coli* for elevating the expression levels of lipase. The molecular weight of expressed lipase was found to be 50 KDa obtained by SDS PAGE [111]. A unique mono- and diacylglycerol lipase from *Penicillium cyclopium* PG37 was cloned and expressed in *Pichia pastoris* GS115. The recombinant lipase showed a highest lipase activity of 40.5 U/ml and stable pH of 6.5–9.5 and 35 °C temperature [112].

Thermostable lipase gene from *Pseudomonas florescens* SIK W1 was isolated and expressed in *E. coli* BL21. From the above study it was found that the expressed enzyme showed highest thermostability [113].

Purification of the crude microbial product is an important step. The microbial product after fermentation is obtained in a very dilute condition. Hence, concentration of the broth is the first step of purification. Different literature reports on purification of protease enzyme are cited below:

Lipase from squid liver was purified initially with ion exchange chromatography followed by octyl-sepharose column [114]. The optimum pH and temperature for enzyme activity was found to be 35-40 ^{o}C temperature and pH 8 with molecular weight of 27 KDa. An extra-cellular lipase was purifiedfrom the *Lactobacillus* sp G5 isolate. The expressed lipase was characterized after ammonium sulphate precipitation and dialysis. Stability of the enzyme was found at pH range 7-9, with temperature range 30 °C-60 °C and the isolate also showed good probiotic properties.

Lipase gene from psychrotrophic bacteria, *Pseudomonas* sp ADT3 was isolated, purified and characterized for lipase production [115]. The enzyme obtained after partial purification by dialysis was found to be active at pH 8.0 and temperature 22 °C. The enzyme showed a molecular weight of 13.9 KDa. Lipase enzyme from *Saccharomyces cervisiae* was isolated and purified by ammonium sulphate followed by dialysis [116]. Then the dialyzed extract was subjected to sephadex G 100 column chromatography. The purified enzyme showed a specific activity of 30 U/mg which was 4.1 times higher than the native.

Bacillus sp, was isolated from soil for lipase production. The lipase enzyme was purified by ammonium sulphate precipitation, dialysis followed by DEAE column (ion exchange chromatography) [117]. The purified enzyme obtained after purification showed a highest activity of 15.24 U/mg.

Lipase from *Beauveria bassiana* was isolated and purified. The purified enzyme showed 9.91-fold increase in production and the specific activity was found to be 20816 U/mg proteins with optimal pH and temperature at 7 °C and 35 °C [118]. Lipase producing psychrotrophic bacteria, *Halomonas* (BRI 8) was isolated from Antarctic water sample. An extracellular cold active lipase enzyme was obtained after purification with ammonium sulphate [119]. The optimum pH and temperature for enzyme catalysis were found to be 7.0 and 10 °C. The enzyme was also found to be stable in various organic solvents.

1.5.6 Immobilization and kinetic study of lipase

Enzymes play a vital role in catalyzing several biochemical reactions. The enzymes are usually unstable, the cost of isolation was high and difficult to recover them after the reaction. Hence the enzymes were immobilized on to solid surfaces which improve the catalytic activities and reusable capacity. Reaction rates are often heavily influenced by physico-chemical conditions and kinetic potentiality which are optimized as they control the rate of reaction. The determination of K_m and V_{max} values of enzyme system was necessary to

determine the kinetic potentiality. In the literature much information is available on general methods and special techniques on immobilization and kinetic study of lipase enzyme.

Lipase from *Candida rugosa* was immobilized onto ferromagnetic azide polyethyleneterepthalate (Dacron) and a comparative study of native and immobilized enzyme was done [120]. From the study it was evident that the immobilized lipase was more efficient for catalyzing the hydrolysis of 4-nitrophenyl esters and showed more thermostability than the native one.

Lipase2 from *Staphylococcus xylosus* was immobilized on $CaCO_3$. The immobilized lipase was found to be stable at 80 °C and produces high yield compared to free enzyme [121]. The immobilized lipase produced ricinoleic acid esters.

Pseudomonas aeruginosa SRT9 lipase was immobilized on n-tri(4-formyl phenoxy) cyanurate [122]. A specific activity of 12307.81 U/mg was obtained with immobilized lipase. An increase in K_m value from native 0.043 mM to 0.10 mM for immobilized lipase was observed with decrease in V_{max} value.

Lipase from *Candida rugosa* was immobilized onto palm-based polyurethane foam (PU) [123]. The immobilized lipase showed thermostability, operational and storage stability.

Lipase from *Candida antarctica* B was immobilized by sol-gel entrapment. The immobilized enzyme showed high activity than free enzyme and the activity remained stable even after reusing 15 times [124].

Ferromagnetic azite Dacron was used for immobilizing lipase of *Pencillium citrinum*. No change in pH or temperature was observed with the free and immobilized enzymes [125]. The K_m values for free and immobilized enzyme were 233 µM and 276 µM. The immobilized enzyme showed good operational stability. AE-cellulose partially modified by palmitoyl residue was used in immobilizing the lipase from fungus *Oospora lactis.* It was shown to be most effective method of immobilization [126].

Lipase from *Enterobacter aerogenes* 13 was immobilized on lignin and poly urethane [127]. The immobilized strain was found to be stable in wide range of pH. The enzyme was found to be highly active at alkaline conditions with pH 8-9 and temperature 30-40 °C. Lipase from *Thermomyces lanuginosus* was immobilized on PVA (poly vinyl alcohol) beads [128]. The operational stability of the immobilized enzyme was found to be at pH 4-7 and temperature 40 °C -80 °C. Lipase from *Mucor miehei* was immobilized onto a PES–NH2 membrane and through the study it was evident that the immobilized enzyme showed better stability and higher reusability than the free enzyme [129].

1.5.7 Lipase mediated ester synthesis

Lipases catalyze different reactions like esterification, transesterification, hydrolysis, aminolysis, acidolysis and regioselective acylation of glycerols resulting in synthesis of peptides, esters, amides and other chemicals. The esters are compounds derived from acid. The esters synthesized by lipase finds applications in numerous fields such as food, detergent, pharmaceutical, leather textile, cosmetic, biodiesel production and paper industries. Short chain fatty acids esters are important aroma compounds used in different flavoring industries, whereas esters of long chain fatty acids are being explored for their use as fuel (biodiesel) and as waxes. Different esters like ethyl, isobutyl, amyl, isoamyl, isopropyl, benzyl, octyl and methyl acetates were frequently used in flavouring and perfumes industries. Some esters act as drug intermediates and nutraceuticals.

Esterification mediated by immobilized lipase from *Rhizopus oryzae* NRRL 3562 synthesized short chain fatty acid esters like methyl butyrate and octyl acetate [130]. Methyl butyrate ester has a fruity odour of pineapple, apple, and strawberry where as octyl acetate has orange flavor which were applied in food and beverage industries as flavoring agents. *Rhizopus oryzae* NRRL 3562 lipase mediated transesterification was carried out with isoamyl alcohol

and vinyl acetate to synthesize isoamyl acetate which is widely used in food and cosmetic industry [131].

Butyl butyrate, a flavor ester was synthesized by transesterification of lipase from *Aspergillus niger*. Optimal conditions for production of butyl butyrate ester were obtained using statistical tools like Face Centered Design (FCD) and RSM. Highest yield at 40 °C with 1:2 ratio of butyric acid and butanol was obtained [132].

Candida rugosa lipase was immobilized on Sepa beads EC-EP for synthesis of amyl caprylate in prescence of isooctane. RSM and 5-level-5-factor CCRD were used to optimize the reaction parameters and the properties of immobilized lipase were found to be pH 7.5; temperature 45 ºC - 47 ºC [133].

Commercial lipase (Steapsin) was immobilized on silica which was pretreated with glutaraldehyde [134]. The immobilized lipase was used in esterification of butanol and ferulic acid for synthesizing butyl ferulate. Lipase from *Pseudomonas cepacia* was used as a catalyst for esterification of carboxylic acid for hydrocinnamic ester synthesis which is widely used in flavor and fragrance industries [135].

Immobilized lipase from *Candida antarctica* (Novoenzyme 435) was used as a catalyst for synthesizing vitamin esters like retinyl- L –lactate by retinol and L-methyl lactate and ascorbyl- L- lactate by esterification of ascorbic acid and L-methyl lactate [136]. In another study it was reported that this lipase was used as a biocatalyst in synthesizing lauryl palmitate, a wax ester by esterification of palmitic acid and lauryl alcohol [137]. Novoenzyme 435 was also used in synthesis of sucrose esters by esterification of palmitic acid and sucrose which can be applied in food industries [138]. There was another report on *Candida antarctica* for synthesis of isopropyl laurate from isopropyl alcohol and lauric acid. This enzyme was found to be highly economical and effective in producing different esters [139].

Lipase from germinated seeds of Jatropa and rice bran was used to synthesize oleic acid ethyl ester by esterification of oleic acid and ethanol. The conditions for the synthesis were optimized using RSM. From the optimized parameters the amount of oleic acid synthesized was 810.77 μmol and 626.92 μmol from lipases of ricebran and germinated Jatropha seed respectively [140].

1.6 Gaps in existing research

The above review states that the production of lipase shows that a variety of microbes have been utilized for its production. Extensive literature review and industrial survey reveals that lipase has captured nearly 70% of the total enzyme market in the world and is gaining much importance. This gives us enough scope to study and develop more efficient process for its production in laboratory scale as well as in industrial scale since each methodology differs with respect to the microorganisms used. These studies have revealed a lot of information on isolation, production, purification and application of lipases. A careful introspection reveals that there is a compelling need to look for more effective strategies like

- To develop co-culture methodology for production of bacterial lipases as the lipase production from co-culture technique was meagerly reported in the literature.
- To develop co-culture methodology for lactic acid bacterial strains which are not yet reported in literature.
- To develop economically viable/cheaper way of production and diverse array of genetically distinct lipases.
- To study the variation in the rate of hydrolysis based on the variation in microbial sources.

1.7 Scope and objective of the work

In an attempt to develop effective strategies for lipase production the present study has the following aims

- Selection of lipase producing microorganisms by single and co-culture methodology and optimization of physical parameters for lipase production from selected microbial sources.
- Strain improvement by mutagenesis
- Heterologous expression of lipase gene for overexpression.
- Purification and kinetic study of lipase.
- Immobilization of lipase.
- Lipase mediated ester synthesis which can be used as flavoring agents in food industries.

Material and Methods

This chapter gives an outline of the materials used and the general experimental procedures adopted for production, expression, purification and application of lipase from single and co-cultures of different lactic acid bacteria proposed in the study.

2.1 Materials:

2.1.1 Microorganism

For present investigation different Lactic acid bacterial strains like *Lactococcus lactis, Lactobacillus brevis* and *Lactobacillus plantarum* were used which were obtained from MTCC (Microbial Type Culture Collection, India).

2.1.2 Substrate

Olive oil was used as substrate for the experiments which was procured from local shops.

2.1.3 Chemicals

The following chemicals have been used in the present investigation.

Peptone, Beef extract, Yeast extract, Glucose, Disodium hydrogen phosphate, Sodium acetate, Triammonium citrate, Magnesium sulphate, Manganese sulphate, Potassium acetate, Agarose, Sodium chloride, Tris base, TEMED, Bromophenol blue, Sodium dodecyl sulphate, Bovine serum albumin – procured from **Himedia, India**.

p-Nitrophenyl palmitate, p- Nitrophenol, Tween-80 – procured from **Sigma chemicals**.

Primers, Restriction enzymes (EcoR1, BamH1 and XmnI), 10x assay buffer, T_4 DNA ligase, DNA polymerase, dNTP's, 1Kb Step up DNA ladder, SDS protein marker (medium range) – procured from **Bangalore Genei, India**.

Amylose resin columns, Plasmid pMALc5X - **New England Biolab**

StrainBL21 (DE3) pLysS for protein expression – **Bionova**

DNA gel Extraction kit - **BioServe**

Copper sulphate, Glycerol, Hydrochloric acid, Triton-X-100, Ferric chloride, Folinciocalteu reagent, Fructose, EDTA - procured from E. **Merck India**.

β-mercaptoethanol, Acrylamide, Bis-acrylamide, Ammonium per sulfate, Glycine, Methanol, Acetic acid, Glacial acetic acid, Acetone, Calcium chloride, Trichloro acetic acid, Di iso propyl ether, Butanol, Tetra acetate, Toulene, 1,4- Dioxane - procured from **Hychem, India**.

Coomassie brilliant blue R-250, N,N,N$'$N$'$ tetra methyl ethylene diamine (TEMED) procured from **SRL, India**.

Ammonium sulphate – procured from **S.D.S.,India**.

Antibiotics - Amoxicillin, Penicillin, Tetracycline, Ampicillin, Streptomycin - Himedia, India.

2-hydroxy benzyl alcohol, 3-hydroxy benzyl alcohol, 4-hydroxy benzyl alcohol, vinyl acetate - **Himedia, India**.

2.1.4 Instruments:

The instruments used in the course of this work are listed below:

Autoclave (Mac Instruments, India): Used for sterilization of the media before inoculation and before disposal of the fermented material.

Balance (Afcoset): For weighing chemicals (mg level).

Laminar air flow cabinet (IAS, India): For inoculation under aseptic condition.

BOD Incubators (Yorko Scientifics, India): Used for growth of micro-organism at constant temperature.

Hot air oven (Yorko Scientifics, India): Used for drying glass wares.

Cooling centrifuge (Eppendorf, India): Used for centrifuging at low temperatures.

pH meter (Elico, India): Used for pH measurement.

Water bath (MAC, India): Used for maintaining constant temperature.

PCR (Applied biosystem): Used for amplification of DNA.

UV- Vis spectrophotometer: (Beckman) For routine optical measurements.

Gel-electrophoresis apparatus: (GE Healthcare Lifesciences) For protein purification purpose.

Gel dock: (G-BOX): For imaging the agarose gels.

Vest frost-20 refrigerator (Celsius refrigerator pvt ltd): For storing primers, vectors at low temperatures.

Millipore unit (Millipore, India): Used as water source for media preparation.

Vortex mixer (MAC instruments, India): Used for gentle mixing of solutions.

Microscope (Olympus, India): Used to detect the microorganisms.

UV-chamber (UVITECH, India): Used for mutational studies.

Spectromax M2 (Molecular devices, California): Multimode reader used for kinetic study of the enzyme.

2.1.5 Medium

The following media were prepared in distilled water and used during the course of present study.

1) Nutrient agar: Used for initial screening of microorganisms it contains

Nutrient broth	20 g/l
Agar	20 g/l

2) MRS medium (so named by its inventorsde Man, Rogosa and Sharpe): Used for maintaining the mother cultures it contains

Peptone	10 g/l
Beef extract	10 g/l
Yeast extract	5 g/l
Glucose	20 g/l

Tween 80	1 ml/l
Disodium hydrogen phosphate	2 g/l
Sodium acetate	2 g/l
Triammonium citrate	2 g/l
Magnesium sulphate	0.2 g/l
Manganese sulphate	0.2 g/l
Agar	15 g/l

3) Egg yolk agar medium: Used for detection of lipolytic microorganisms. It contains

Peptone	40 g/l
Disodium phosphate	5 g/l
Monopotassium phosphate	1 g/l
Sodium chloride	2 g/l
Magnesium sulphate	0.1 g/l
Glucose	2 g/l
Hemin	0.005 g/l
Agar	25 g/l

4) Spirit blue agar medium: Used for detection of lipolytic microorganisms. It contains

Casein enzymic hydrolysate	10 g/l
Yeast extract	5 g/l
Spirit blue	0.150 g/l
Agar	17 g/l

5) Tributyrin agar medium: Used for isolation of lipase producing microorganisms it contains

Peptone	5 g//l
Beef extract	3 g/l
Tributyrin	10 ml/l

Agar	20 g/l

6) Lipase assay medium: Used for production of lipase from the strains it contains

Peptone	5 g/l
Yeast extract	3 g/l
Sodium chloride	2.5 g/l
Magnesium sulphate	0.5 g/l
Olive oil	2 ml/l

7) Tryptone broth: Used for Indole test. It contains

Casein enzymatic hydrolysate	10 g/l
Sodium chloride	5 g/l

8) MRVP broth (Methyl red Vogesproskeur): Used for MRVP test. It contains

Peptone	7 g/l
Glucose	5 g/l
Potassium phosphate	5 g/l

9) Simmon's citrate agar medium: Used for Citrate test. It contains

Sodium citrate	2 g/l
$MgSO_4$	0.2 g/l
Ammonium dihydrogen phosphate	1 g/l
Dipotassium hydrogen phosphate	1 g/l
Sodium chloride	5 g/l
Bromothymol blue	0.08 g/l
Agar	15 g/l

10) Trypticase Soy agar: Used for Oxidase test. It contains

Tryptone	15 g/l
Soytone	5 g/l

Sodium chloride	5 g/l
Agar	15 g/l

11) Nitrate broth: Used for Nitrate test. It contains

KNO_3	1 g/l
Peptone	5 g/l
Beef extract	3 g/l

12) Starch agar medium: Used for Starch hydrolysis test. It contains

Starch	20 g/l
Beef extract	3 g/l
Peptone	5 g/l
Agar	15 g/l

13) Mueller-Hinton agar medium: Used for Antibiotic sensitivity test. It contains

Beef infusion solids	4 g/l
Starch	1.5 g/l
Casein hydrolysate	17.5 g/l
Agar	15 g/l

14) Luria Bertani medium: Used for growth of competent *E.coli* cells

Tryptone	10 g/l
Yeast extract	5 g/l
Sodium chloride	10 g/l

2.1.6 Buffer and Other Reagents

(All solutions were made in deionized water)

2.1.6.1 For lipase estimation

(A) 0.1M Tris Cl buffer (pH 8.2)

Tris base	1.576 g/l

1.576 g Tris base was added to 50 ml distilled water. The pH was adjusted to 8.2 with 6N HCl and the volume was made upto 100ml with distilled water.

(B) 420 μM p-nitrophenyl palmitate

p-nitrophenyl palmitate	0.0135 g
Sodium dodecyl sulphate	0.017 g
Triton X-100	1 ml

All were added to 100ml beaker and the volume was made up with distilled water.

(C) 0.2 (M) Glycine NaOH Buffer pH 8.0

(D) Bovine Serum Albumin (BSA) stock solution (10 mg/ml)

(E) 2% Casein solution

(F) 2% Sodium Potassium Tartarate solution (Reagent-B_2)

(G) 1% $CuSO_4.5H_2O$ solution (Reagent-B_1)

(H) Alkaline solution (Reagent A)

$NaCO_3$	20 g/l
NaOH	4 g/l

(I) Folin Reagent

Folin ciocalteau reagent: H_2O (1:1)

(J) 5% (w/v) Trichloro Acetic Acid (TCA) solution

2.1.6.2 For Biochemical studies

a) Kovac's reagent: For indole test

P-dimethylaminobenzaldehyde	15 g
Isoamyl alcohol	150 ml
HCl	75 ml

b) Barrit's reagent: For MRVP test

VP 1 reagent	5 g
	(α-naphthol dissolved in 95 ml absolute alcohol)
VP 2 reagent	40% KOH solution

c) Nitrate test reagent: For determination of nitrate reduction by bacteria

Zinc chloride	20 g/l
Starch	4 g/l
Potassium iodide	2 g/l

d) Iodine solution: For starch hydrolysis test

Iodine	1 g/300 ml
Potassium iodide	2.9 g/300 ml

2.1.6.3 For SDS-PAGE Gel –electrophoresis

a) 1.5M Tris Buffer pH 8.8

Tris base	181.71 g/l

pH 8.8 adjusted with concentrated HCl, using pH meter.

b) 1M Tris Buffer pH 6.8

Tris base	60.57 g/l

pH 6.8 adjusted with concentrated HCl, using pH meter.

(c) Acrylamide and Bisacrylamide Solution

Acrylamide	60 g/l
Bisacrylamide	1.6 g/l

This solution was stored in dark at 4 ^{o}C to prevent slow polymerization and hydrolysis. Fresh solution should be prepared for use every month.

(d) 10% Ammonium persulphate solution.

(e) N, N, N, N-Tetra Methyl Ethylene Diamine (TEMED): commercially available.

(f) 10% sodium dodecyl sulphate

(g) Sample loading buffer

Tris buffer 0.5 M pH 6.8

SDS	2% (w/v)
Glycerol	10%
Bromophenol blue	0.1%
β-mercaptoethanol	5%

(h) Gel electrophoresis buffer (pH 8.3)

Tris base	6 g/l
Glycine	28.4 g/l
SDS	0.1 %

(i) Resolving gel (12%)

1.5 M Tris buffer pH 8.8	2.5 ml
Acrylamide + bisacrylamide	4 ml
10% SDS	0.1 ml
TEMED	25 µl
10% APS	45 µl
Distilled H_2O	3 ml

(j) Stacking gel (5ml)

0.5 M Tris buffer pH 6.8	0.63 ml
Acrylamide + bisacrylamide	1.0 µl
10% SDS	50 µl
TEMED	10 µl
10% APS	45 µl

Distilled H_2O	3.15 ml

(k) Staining solution

Methanol: H_2O (1:1, v/v)	90 ml
Glacial acetic acid	10 ml
Coomassie Brilliant Blue (R-250)	0.25 g

(l) Destaining solution

Methanol	90 ml
Glacial acetic acid	10 ml

Final volume adjusted with distilled H_2O.

2.1.6.4 For Heterologous expression

(a) For PCR reaction (25 µl)

Buffer	2.5 µl
dNTP's	2.0 µl
Taq	1 µl
Primer Forward	1 µl
Reverse primer	1 µl
DNA	1 µl
Water	16.5 µl

(b) Gel for agarose gel electrophoresis

Agarose	0.4 gms
1xTAE	40 ml
Ethidium bromide	3 µl

(c) For vector digestion (10 µl)

Vector	3 µl
10 x assay buffer	1 µl
Bam H1	2 µl
Xmn1	1 µl
Water	3 µl

(d) For insert digestion (10 µl)

10 X assay buffer	1 µl
Bam H1	2 µl
Xmn1	1 µl
Water	3 µl
PCR product	3 µl

(e) For ligation (10 µl)

Digested vector	2 µl
Digested insert	4 µl
T_4 DNA ligase	2 µl
Water	2 µl

(f) For preparation of competent *E.coli* cells using Calcium chloride

1 M $CaCl_2$ 111 g/l

(mix calcium chloride ($CaCl_2$) in distilled water and filter through 0.22µ filter).

0.1 M $CaCl_2$

(Add 100 ml of calcium chloride in 900 ml of distilled water).

50% Glycerol 50ml glycerol in 50ml of water

2.1.6.5 For Esterification reactions

Lipase	100 mg
Substrate	50 mg
Solvent	900 µl
Acetylating agent	100 µl

2.2 Methods:

2.2.1 Strain maintenance in slants:

The strains were maintained on MRS agar slants at 4 °C. The strains were sub cultured routinely.

2.2.2 Isolation of lipase producing organisms

All the strains were grown on tributyrin agar, egg yolk agar and spirit blue agar by taking a loop full of culture on to the agar plates. The lipase producing organisms were selected on the basis of formation of zone of hydrolysis around the colonies on tributyrin, egg yolk agar and spirit blue agar plates.

2.2.3 Cell counting method:

Using haemocytometer the actual count of bacterial cells was monitored to be approximately 2.02×10^6cells/ml of *Lactococcus lactis*, 1.04×10^6 cells/ml of *Lactobacillus brevis*, 1.20×10^6 cells/ml of *Lactobacillus plantarum* respectively.

2.2.4 Fermentation process:

Fermentation was carried in 250 ml of Erlenmeyer flasks each containing 50 ml of a medium composed of peptone (0.5%), yeast extract (0.3%), NaCl (0.25%), $MgSO_4$ (0.05%) and olive oil (substrate). Then to each flask, 2 ml inoculum of each mentioned bacterium was added for the single cultures. For the co-culture medium the inoculum should be in the ratio of 1:1 to make it 2 ml. After incubation, the cell-free supernatant was obtained by centrifugation

at 7860 rpm at 4 °C for 20 minutes and the extracellular lipaseactivity of the fermented broth was determined. Whereas the cell mass concentration was evaluated as dry weight in (mg/ml) from the pellet directly by weighing the biomass after drying. This showed the growth with the dominance of the respective bacterium in response to the lipase yield after the optimization.

2.2.5 Determination of total protein by Lowry method [141]

Protein solution of 0.5 ml was taken and reagent C was added to it and mixed thoroughly. After 10 minutes at room temperature, Folin ciocalteau (0.5 ml) was added and mixed well. Then the absorbance was taken at 750 nm against a blank after 30 minutes at room temperature, where the protein solution was replaced by deionised water or appropriate buffer. A standard curve was prepared with various concentrations of BSA solution to find out the unknown concentration of the protein hydrolysate.

2.2.6 Lipase assay

1. 0.05 to 0.50 ml of 0.5 mM (millimolar)-nitrophenol standard solution was taken into ten individual test tubes and each tube was diluted with 5 ml of 0.1 M TrisCl buffer, pH 8.2 which yields a standard curve of *p*-nitrophenol/ml [142].

2. The absorbance was measured at 410nm using 0.1 M TrisCl buffer, pH 8.2, as a blank, and a standard curve by plotting absorbance versus the *p*-nitrophenol concentration in each tube.

3. For each lipase activity assay, 2.5 ml of 0.1 M TrisCl buffer, pH 8.2, and 2.5 ml of 420 μM *p*-nitrophenyl palmitate substrate solution was taken into a 15- to 20-ml test tube.

4. 1 ml of water was added to the reagent blank.

5. 1 ml of lipase solution was added into the next substrate-containing tube to initiate the reaction. The tubes were vortexed properly and transferred into sterile cuvette. The absorbance was recorded every minute for up to 15 min.

6. *p*-nitrophenol standard curve was used to convert absorbance to mMsubstrate hydrolyzed by using the following formula.

Mmol *p*-nitrophenol/ml reaction mixture = ($A410 - y$ intercept)/ (slope × 6 ml reaction mixture)

7. Lipase activity was determined by constructing a curve of concentration of nitrophenol (mM) released versus reaction time. A tangent was drawn to the initial portion of the progress curve to obtain initial reaction rates ($v0$ in mM/min) by the following formula

$$v0 = \text{slope} = (y2 - y1)/(x2 - x1)$$

8. The specific activity (sp. act.) of the lipase was determined by the following formula

sp. act. = $v0 \div [a$ mg protein/6 ml reaction volume]

Where a is ml protein added to 6 ml reaction mixture, and units are μmol/(min × ml protein), which is equivalent to U/ml protein.

2.2.7 Cell dry weight determination

1. The eppendorf was pre-weighed.

2. 1.5 ml of cell broth was added into weighed eppendorf and centrifuged at 14,000 rpm for 10 min.

3. Supernatant was discarded and pellet was dried by freeze dryer.

4. The eppendorf with dried cell pellet was weighed to measure dry weight.

2.2.8 Mutagenesis

1. Bacterial suspension of 1 ml each was taken in test tubes.

2. One control for each was kept aside and the rest of the tubes were exposed to UV for 30 minutes to 120 minutes at an interval of 30 minutes at an intensity of 254 nm irradiation.

3. Immediately after exposure to UV, 20 microlitres from 1ml of the sample was spread on the tributyrin agar plate and incubated at their respective optimum temperatures.

4. After incubation, the cells were inoculated into lipase assay medium.

5. The enzyme activity was measured at 410 nm using *p*-nitrophenyl palmitate as substrate.

2.2.9 Isolation of genomic DNA (Protocol from Miniatris)

1. Genomic DNA was isolated using standard DNA extraction procedure.

2. Isolated organisms were grown in 5 ml LB (Luria Bertani) medium at 37 ºC for overnight.

3. The culture was centrifuged at 14000 rpm for 5 minutes and the pellet was lysed using GET buffer (Glucose-20%, Tris buffer-50mM and EDTA- 50mM) followed by lysozyme treatment for 30-40 minutes at 37 ºC.

4. Subsequently 10% SDS was added to the lysozyme treated culture and kept at 37 ºC for 1-2 hours.

5. Extraction was done by adding equal volumes of PCI (Phenol: Chloroform: Isoamyl alcohol) and ethanol treatment was done and the precipitate was dissolved in 30 µl of Tris-EDTA (TE) buffer. The obtained DNA was run on agarose gel electrophoresis.

2.3.0 Isolation of plasmid DNA using Mini prep method (Protocol from Miniatris)

1. A colony of plasmid pMALc5X was inoculated into 50 ml of LB broth containing ampicillin antibiotic and incubated overnight at 37 ºC.

2. 1.5 ml of culture was taken into centrifuge tube and centrifuged at 7860 xg for 30 seconds at 4 ºC.

3. Supernatant was removed and the pellet was dried. The pellet was resuspended in 100 µl ice cold alkaline lysis solution-1 by vortexing vigorously. 200 µl of alkaline lysis solution-2 was added into the tube and mixed.

4. 150 µl of alkaline lysis solution-3 was added through bacterial lysate and stored on ice for 3-5 minutes and centrifuged for 5 minutes at 7860 xg at 4 ºC.

5. Two volumes of ethanol were added to precipitate nucleic acids and the mixture was allowed to stand for 2 minutes at room temperature and centrifuged. 1 ml of 70% ethanol was added to pellet and centrifuged at 7860 xg for 2 minutes at 4 ºC.

6. Supernatant was removed and pellet containing nucleic acids was dissolved in 50 µl of TE containing 20 µg/ml DNase free RNase A. The solution was gently vortexed and the DNA was stored at -20 ºC.

2.3.1 Gel elution method

1. The gel containing the DNA fragment of interest was excised in Gel doc after agarose gel electrophoresis and stored at 4 ºC in pre weighed eppendorf.

2. The weight of gel was taken and 3 volumes of buffer GA was added (for each gram of the gel) and incubated at 50 ºC for 10 minutes till agarose melts completely and mixture turns orange to purple colour.

3. 10 µl of 3M (molar) sodium acetate (pH 5) was added and kept for 5 minutes incubation till colour becomes pale yellow.

4. The suspension was transferred into Bioserve column and centrifuged at 6500 rpm for 1 minute. The supernatant was removed and 500 µl of buffer GA was added and centrifuged at 12000 rpm for 1minute to remove the reminents of agarose.

5. The column was washed with 500 µl of buffer GB and centrifuged at 12000 rpm for 1minute.

6. Now the column was spinned at 12000 rpm for 3 minutes and kept at room temperature.

7. The column was placed in 1.5 ml centrifuge tube and 30 µl of buffer GC or water was added and centrifuged at 12000 rpm for 1minute and kept at room temperature for 10 minutes and the resulting elute containing expected DNA was run on agarose gel.

2.3.2 Preparation of competent *E. coli* cells (Protocol from Miniatris)

1. Strain BL21 (DE3) pLysS was streaked on LB plate containing 100 µg/ml ampicillin and incubated for overnight.

2. One colony was picked up from the LB plate and inoculated into 10 ml of LB broth and incubated at 37 ºC over night.

3. The cells were allowed to grow till the $O.D_{600}$ was 0.4.

4. The cells were transferred to centrifuge tubes and placed on ice for 20 minutes and centrifuged at 4 ºC for 10 minutes at 2500 rpm.

5. The cells were transferred into 50 ml of propylene tubes containing 30 ml of 0.1 M $CaCl_2$ and incubated on ice for 30 minutes.

6. The suspension was centrifuged at 4 ºC for 10 minutes at 2500 rpm.

7. The supernatant was removed and the cells were resuspended in 8 ml of cold 0.1 M $CaCl_2$ containing 15% glycerol and stored at -80 ºC.

2.3.3 Purification of fusion protein

1. Overnight cultures containing the fusion plasmid were inoculated into LB broth containing glucose and ampicillin.

2. The cells are grown till the $O.D_{600}$ was 0.5. The cells were harvested by centrifugation at 4000 xg for 20 minutes and supernatant was discarded. The pellet was suspended in 25 ml column buffer.

3. The samples were placed in an ice water bath sonicated in short pulses of 15 seconds.

The release of protein was checked using the Lowry's method.

6. The samples were centrifuged at 20,000 xg for 20 minutes. Pellet was discarded. The supernatant contains the crude extract which was diluted with column buffer in 1:6 ratio.

7. The amylose resin was poured in a 2.5 x 10 cm columnand the column was washed with 5 volumes of column buffer.

8. The diluted crude extract was poured into the column.

9. The fusion protein was eluted with Column Buffer + 10 mM maltose. 10 to 20 fractions of 3 ml each (fraction size = 1/5th column volume) was collected. The fusion protein started to elute within the first 5 fractions, and was detected by UV absorbance at 280 nm or Lowry's method.

2.3.4 SDS-PAGE Gel Electrophoresis

Electrophoresis was carried out in polyacrylamide gel containing SDS according to the method of Laemmli [143]. The slab gel apparatus consisted of two gel layers- a stacking gel of 5% polyacrylamide and a resolving gel of 12% polyacrylamide. Protein samples were mixed with sample loading buffer at the ratio of 1:1 (v/v). Then the samples were loaded in the wells of the gel. After the completion of electrophoresis, the gel was stained in gel staining solution for 2-3 hours. It was then destained with destaining solution overnight. After proper destaining, photograph of the bands was taken. The molecular weight of the fractions was then determined by comparing with the standard marker proteins.

2.3.5 Lyophilization

1. The concentration of the samples was calculated before lyophilization.

2. The samples were stored at -20 ºC for few minutes and kept at -80 ºC for overnight.

3. The samples were centrifuged at 2000 rpm for 5 minutes at 4 ºC. Allow the temperature of the vaccum pump to attain -80 ºC.

4. Then the pump was switched on and samples were allowed to run in lyophilizer for 4 hours.

5. The samples were then recovered in powdered form and 500 µl of distilled water was added for quantification of the samples.

Selection and optimization of the physical parameters for lipase production from Lactic acid bacteria by classical and statistical methods.

3.1 Introduction

Metabolic or biochemical processes are influenced by different parameters of the environment in which they occur [144]. A biological process in which various useful products are formed as a result of microbial metabolism is called fermentation. At the same time it is also a chemical process, hence the name biochemical process. By manipulating the parameters like change of temperature, pH, inoculum volume, substrate volume and incubation period that govern the metabolic processes of the microorganisms the production rate can be increased to industrial level [145-147]. This chapter deals with the study of some environmental parameters for maximum lipase production by different single cultures like *Lactococcus lactis, Lactobacillus brevis* and *Lactobacillus plantarum* and co-cultures like *Lactococcus lactis + Lactobacillus brevis, Lactococcus lactis + Lactobacillus plantarum* and *Lactobacillus brevis+ Lactobacillus plantarum.*

The fermentation conditions vary widely from species to species for each of the organism. So, it becomes very important to know the environmental conditions of any isolated microorganism for maximum enzyme production. There is a report [148] that *Pseudomonas gessardii* strain isolated from oil spilled soil from vegetable oil processing factories was screened for their ability to produce lipase. Maximum enzyme activity was achieved when the organism was cultured in medium supplemented with 1% protease peptone at pH 7, temperature 37 ^{o}C and 48 hrs of incubation. The production of potential alkaline lipase by *Pseudomonas aeruginosa* KM110 sp. was optimized [149] using olive oil as substrate. 3-fold

enhanced lipase production (0.76 U/ml) was achieved after improving the conditions of production medium. The enzyme exhibited maximum lipolytic activity at 45 ^{o}C temperature and pH 7-10.

It is also reported [150] that *Bacillus licheniforms* isolated from the oil mill soil produced maximum lipase production of 16.23 U/ml when selenium was used as a media supplement. Similarly 17.29 U/ml of lipase production was found with nickel as a substrate. 12.43 U/ml and 15.38 U/ml of lipase yield was found with urea and ammonium nitrate. They showed that the physicochemical parameters and the lipase production are interdependent.

Different parameters like temperature, pH, carbon, nitrogen sources, effect of metal ions and detergents were optimized for maximum lipase production by the *Bacillus* sp. MPTK 912 and found that the optimum pH of 8, temperature 35 ^{o}C, glucose and peptone as carbon and nitrogen sources in the production medium showed enhanced lipase production [151]. Various physicochemical parameters such as pH, temperature, incubation period, volume ratio, agitation and effect of different carbon and nitrogen sources were studied in order to determine the optimum conditions for lipase production from *Fusarium oxysporum*, a fungal strain and maximum lipase activity was reported as 16.0 U/ml [152]. They worked on 64 fungal strains and this organism showed maximum activity. 53 strains of bacteria were isolated from oil contaminated soil collected in South Korea and screened for lipase production [153]. 16s rRNA sequencing was performed for the organism which showed clear zone on Rhodamine-B agar media and was reported as *Acenitobacter* sp. Maximum activity was found at pH 6.0, temperature of 25 ^{o}C and incubation of 12 hrs. In addition, increased enzymatic production was obtained when the organisms were cultured in medium supplemented with 1% sucrose as the carbon source.

From the literature, it is clear that different parameters have some individual effect on the microorganisms in their growth and enzyme production. The effect varied from species to

species. So there is immense need to investigate the effect of different physical parameters on lipase production from different single and co-cultures of lactic acid bacteria. In this study, both classical and statistical methods were used for optimizing the parameters.

3.2 Composition of the medium for maintaining mother culture

MRS agar was used to maintain mother culture with composition of Peptone (10 g), beef extract (10 g), yeast extract (5 g), glucose (20 g), Tween 80 (1 ml), $Na_2 HPO_4$ (2 g), sodium acetate (2 g), triammonium citrate (2 g), $MgSO_4.7H_2O$ (0.2 g), $MnSO_4.4H_2O$ (0.2 g), agar (15 g) and distilled water (1 lt).

3.3 Lipolytic Activity

The organisms were grown on tributyrin agar media containing peptone (5 g), beef extract(3 g), tributyrin (10 ml), agar-agar (20 g) and distilled water (1 lt). The formation of a clear zone of hydrolysis around the colony on the plate indicated lipolytic microbes.

3.4 Protein estimation and Enzyme assay

The total protein content was estimated by Lowry's method using Bovine serum albumin as standard. The lipase assay was performed spectrophotometrically using *p*-nitrophenyl palmitate as substrate. The *p*-nitro phenol was liberated from *p*-nitrophenyl palmitate by lipase mediated hydrolysis [154]. The assay mixture contained 2.5 ml of 420 µm *p*-nitrophenyl palmitate, 2.5 ml of 0.1 M Tris-HCl (pH -8.2) and 1ml of enzyme solution. It was incubated in water bath at 37 ^{o}C for 10 min and *p*-nitrophenol was liberated from *p*-nitrophenyl palmitate by lipase mediated hydrolysis imparting a yellow color to the reaction mixture. After incubation, the absorbance was measured at 410 nm. Absorbance of control was also recorded. One unit (U) of lipase was defined as the amount of enzyme that liberates one micromole of *p*-nitro phenol per minute under the assay conditions [155].

3.5 Optimization of fermentation process

Optimum condition for the production of lipase by lactic acid bacteria was determined with respect to incubation period, temperature, pH, substrate volume and inoculum volume, by varying the parameters one at a time.

Lipase assay medium was used for production of lipase from the strains. The extracellular lipase production was carried out in 250 ml Erlenmeyer flasks each containing 50 ml of a medium composed of peptone (0.5%), yeast extract (0.3%), NaCl (0.25%), $MgSo_4$ (0.05%) and olive oil (substrate). The microorganisms used for monoculture and co-culture in this experimentation are given below in Table 1. The bacterial inoculum in co-culture was used in the ratio of 1:1. After incubation, the cell-free supernatant was obtained by centrifugation at 7860 rpm at 4 ^{o}C for 20 min and the extracellular lipase activity of the fermented broth was determined.

3.6 Results

3.6.1 Effect of different substrates on lipase production

To study the effect of different substrates on lipase production, different readily available, moderately cheaper non-edible oils were used as a substrate in the medium and relative growth was measured. Among different oils used olive oil showed good growth compared to other oil used as a substrate in the fermentation media as shown in Figure 3.1. The data represented in the table was obtained from experiment done in triplicates.

(a)

(b)

(c)

(d)

(e)

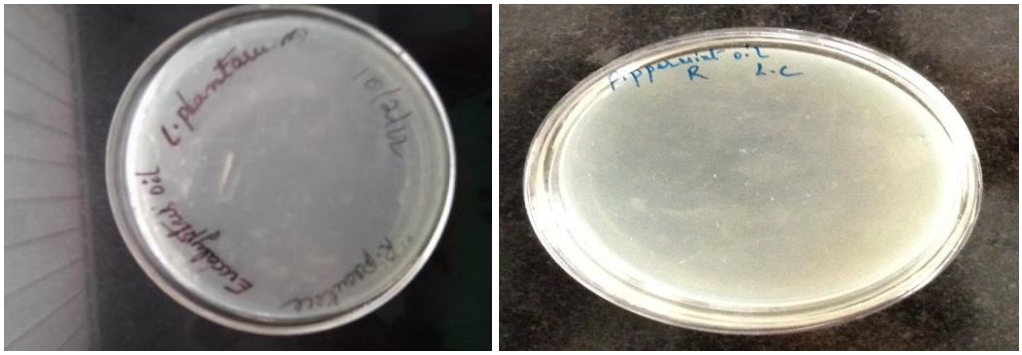


Figure 3.1: Relative growth of microorganisms on different substrates

(a) Petriplates showing no growth in camphor oil (b) good growth in rose oil (c) less growth in lemon oil (d) dense growth in olive oil (e) no growth in eucalyptus and peppermint oil as substrate

Table-3.1: Effect of substrate on lipase production

S. no	Substrate	Relative growth		
		L. lactis	*L. brevis*	*L. plantarum*
1	Peppermint oil	-	-	-
2	Camphor oil	-	-	-
3	Eucalyptus oil	-	-	-
4	Lemon oil	+	+	+
5	Rose oil	+	+	++
6	Olive oil	++	++	++

Where - = no growth, + = moderate, ++ = good growth

3.6.2 Effect of incubation period on lipase production

To study the effect of incubation time on lipase production 250 ml flasks with 100 ml of lipase assay medium were incubated at 37 ^{o}C with 2 ml inoculum for different incubation periods of 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs respectively. After incubation, the lipase activity was measured.

After assay, it was observed that 48 hrs incubation was the optimum fermentation time for co-culture of *L. brevis* + *L. plantarum* and 72 hrs for co-cultures of *L. lactis* + *L. brevis* and *L. lactis* + *L. plantarum*as shown in Figure 3.2.

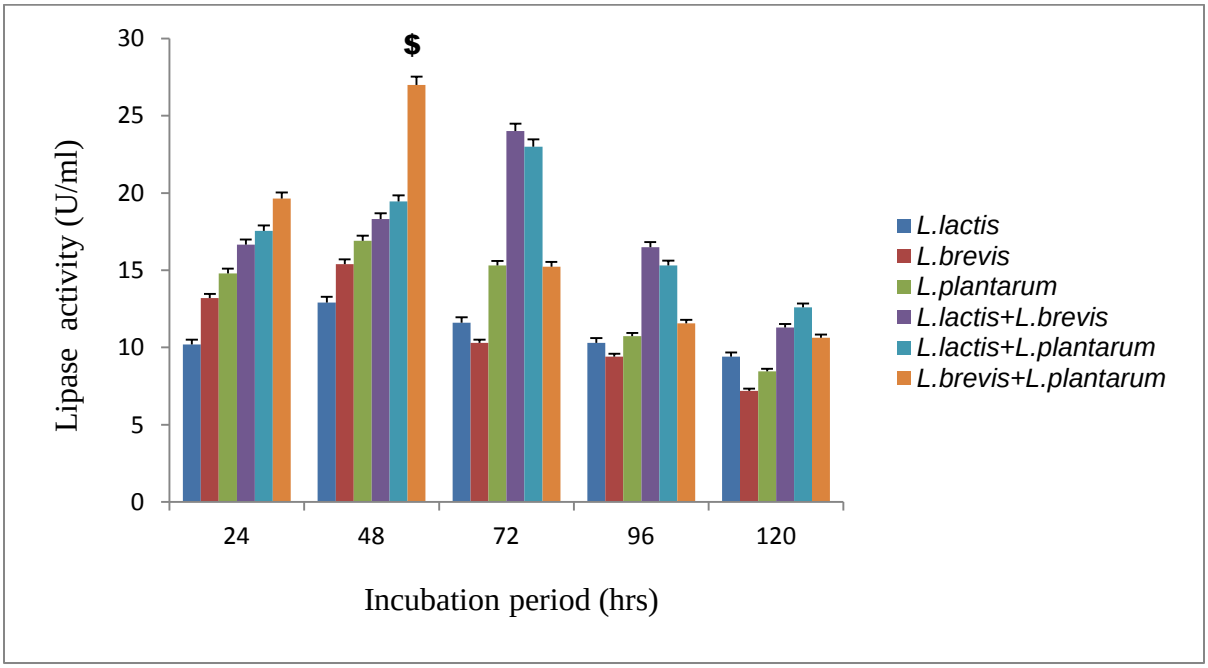


Figure 3.2: Effect of incubation period on enzyme production

Data was mean standard deviation of 9 replicates from 3 experiments ($p \leq 0.05$). $^{\$}$ represents maximum enzyme activity obtained at incubation period 48 hrs.

3.6.3 Effect of initial pH on lipase production

For optimization of lipase production, pH plays an important role. Thus for the present study the initial pH of the medium was varied from pH 5 to 6.5 at an interval of 0.5. Each 250 ml Erlenmeyer flasks were adjusted with suitable pH followed by sterilization which was then

inoculated with 2 ml inoculum. From the results as shown in Figure 3.3 it was concluded that maximum lipase activity was observed for co-culture of *L. plantarum* + *L. brevi*s at pH 5.0 and for co-cultures of *L. lactis* + *L. brevi*s and *L. lactis* + *L. plantarum* it was at pH 5.5.

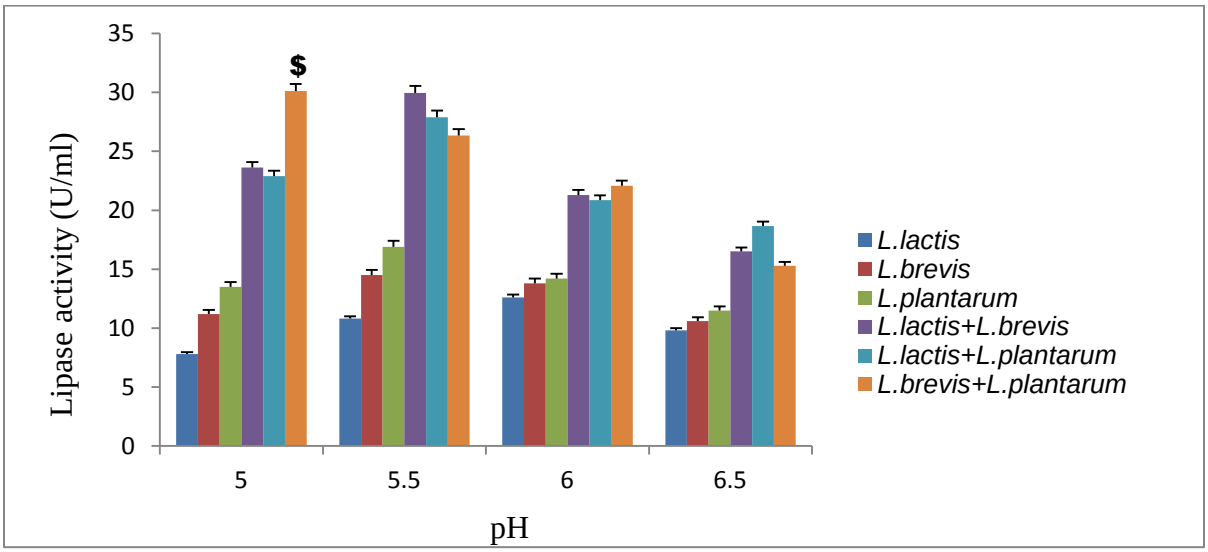


Figure 3.3: Effect of initial pH on enzyme production

Data was mean standard deviation of 9 replicates from 3 experiments ($p \leq 0.05$).

$ represents maximum enzyme activity obtained at pH 5

3.6.4 Effect of temperature on lipase production

Temperature plays an important role in the metabolic regulation of the microorganisms. Temperature stability studies of the above mentioned organisms were done by maintaining incubation at different temperatures of 25 ^{o}C, 30 ^{o}C, 35 ^{o}C and 40 ^{o}C. Lipase activities of the supernatant were analyzed.

Figure 3.4 depicts that the maximum lipase activity was found around at 30 ^{o}C for *L. brevis* + *L. plantarum* and 35 ^{o}C for *L. lactis* + *L. brevis* and *L. lactis* + *L. plantarum* respectively.

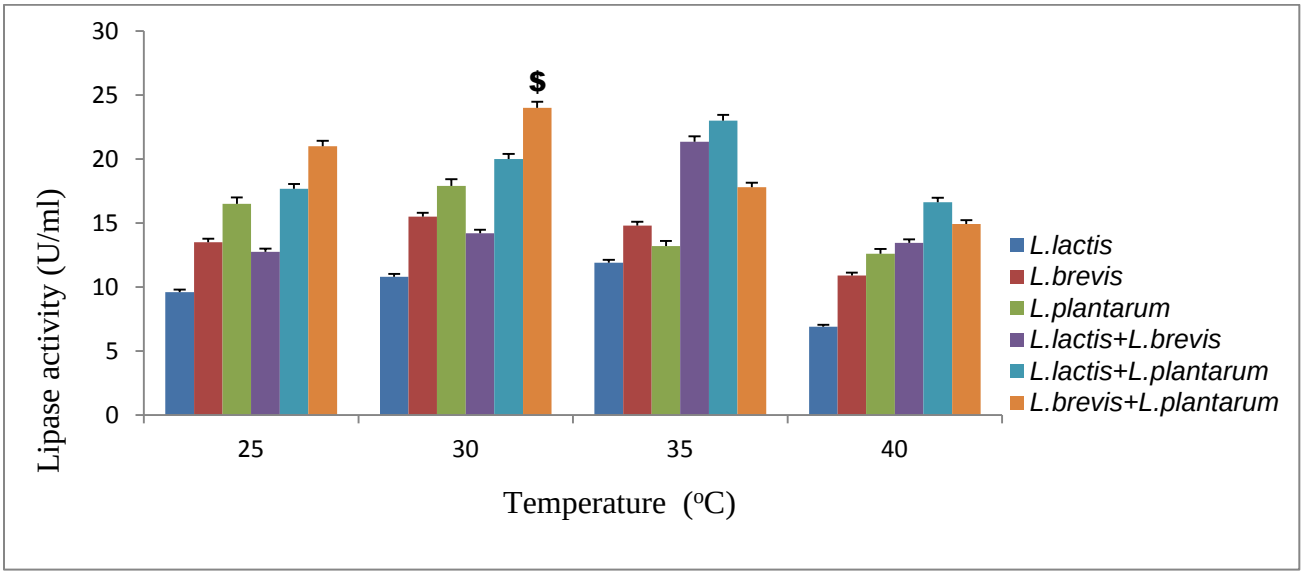


Figure 3.4: Effect of temperature on enzyme production

Data was mean standard deviation of 9 replicates from 3 experiments ($p \leq 0.05$). $^{\$}$ represents maximum enzyme activity obtained at temperature 30 ^{o}C.

3.6.5 Effect of inoculum concentration on lipase production

Inoculum concentration plays an important role in optimization of lipase production. For the present study inoculum concentration was varied from 0.5-2.5 ml with an interval of 0.5. After incubation, the lipase activities of the supernatant were estimated.

All the co-cultures showed maximum production with 2 ml of inoculum as shown in Figure 3.5.

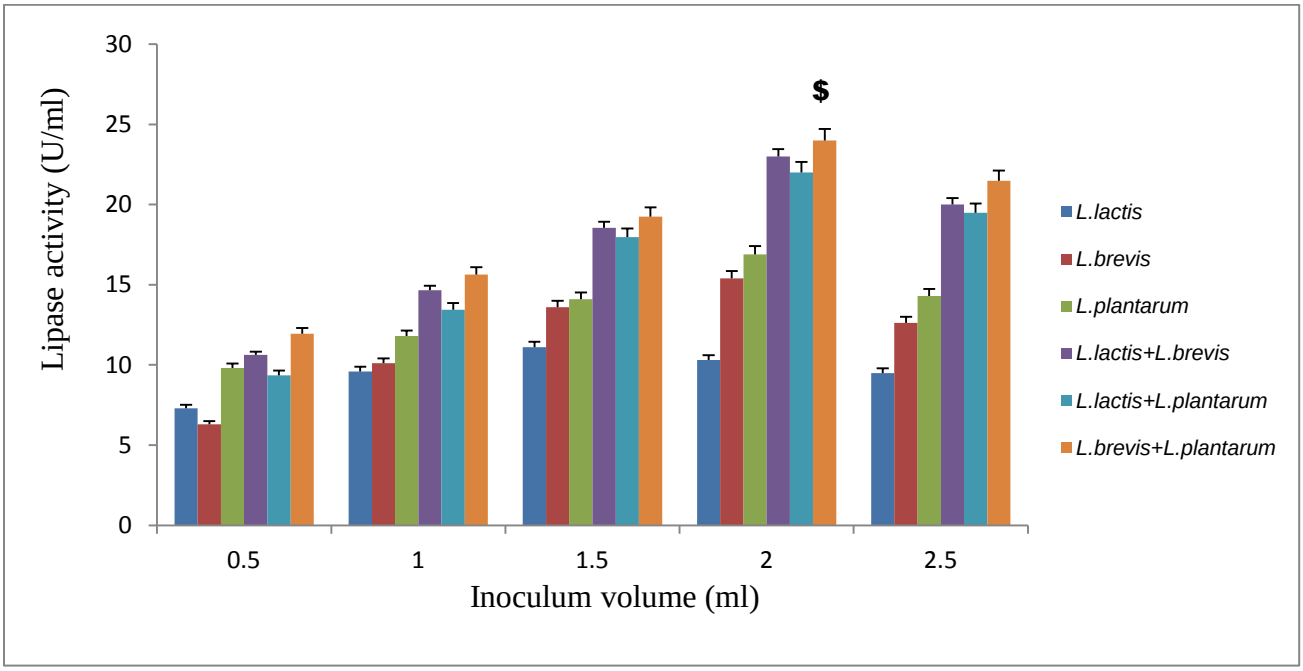


Figure 3.5: Effect of inoculum concentration on enzyme production

Data was mean standard deviation of 9 replicates from 3 experiments ($p \leq 0.05$). $^{\$}$ represents maximum enzyme activity obtained with 2 ml of inoculum.

3.6.6 Effect of substrate volume on lipase production

To study the effect of substrate volume, olive oil was used as a substrate in a range of 0.5 ml-2.5 ml at an interval of 0.5.

Maximum enzyme activity was obtained at 2 ml of substrate volume as shown in Figure 3.6.

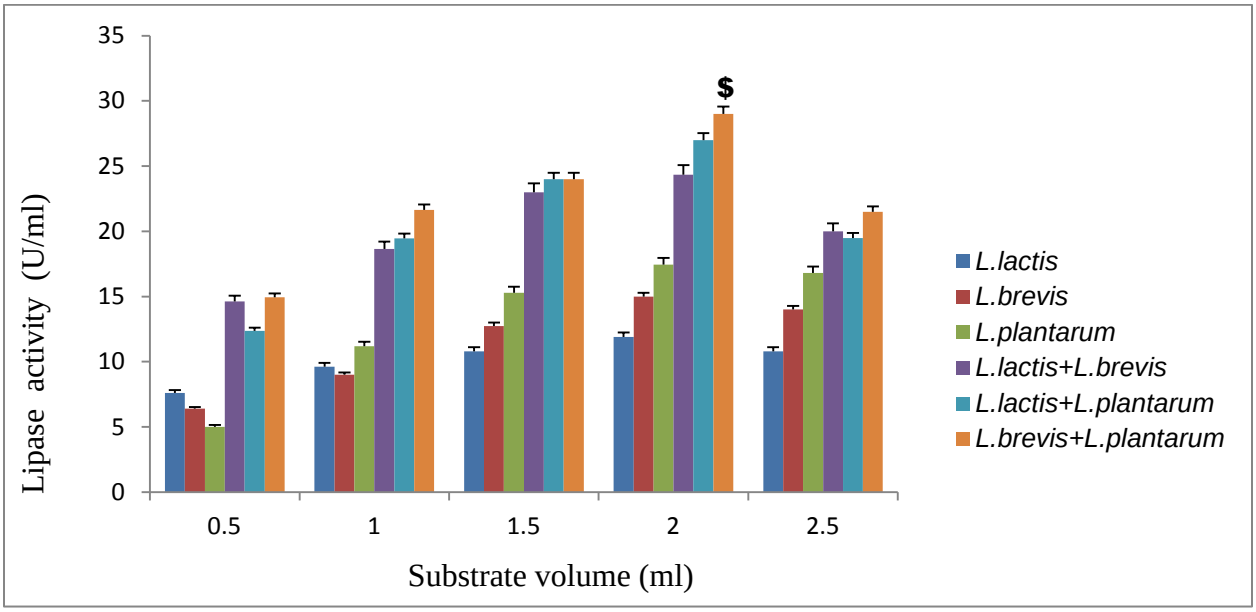


Figure 3.6: Effect of substrate concentration on enzyme production

Data was mean standard deviation of 9 replicates from 3 experiments ($p \leq 0.05$). $^{\$}$ represents maximum enzyme activity obtained with 2 ml of substrate.

3.6.7. Dry cell weight determination

The parent single bacterial cultures of *L. lactis*, *L. brevis*, *L. plantarum* and co-cultures like *L. lactis* + *L. brevis*, *L. lactis* + *L. plantarum* and *L. brevis* + *L. plantarum* were centrifuged at 13,000 rpm for 15 mins at 4 ^{o}C. The pellet was suspended in distilled water and centrifuged. Then the cells were dried and weighed. The biomass was expressed as mg of cell dry weight per milliliter (mg cell dry wt/ml). The dry weight obtained for the parent single and co-cultures were shown in Table 3.2.

Table 3.2: Dry weight of parent single and co-cultures

time (in hrs)	*L. Lactis*		*L. brevis*		*L. plantarum*		*Lc + LBb*		*Lc + LBp*		*LBb+ LBp*	
	Dry weight (mg)	Enzyme activity (U/ml)	Dry weight (mg)	Enzyme activity (U/ml)	Dry weight (mg)	Enzyme activity (U/ml)	Dry weight (mg)	Enzyme activity (U/ml)	Dry weight (mg)	Enzyme activity (U/ml)	Dry weight (mg)	Enzyme activity (U/ml)
12	0.155± 0.002	8.4± 0.11	0.17± 0.003	12± 0.15	0.2± 0.01	13.4± 0.11	0.39± 0.003	16.7± 0.11	0.4± 0.001	19± 0.15	0.82± 0.001	27± 0.15
24	0.224± 0.003	10.± 0.15	0.24± 0.002	13± 0.05	0.3± 0.003	14.8± 0.10	0.42± 0.01	19.7± 0.15	0.45± 0.001	25.4± 0.12	0.86± 0.001	29.64
36	0.249± 0.001	***13± 0.11**	0.28± 0.002	15.2± 0.26	0.3± 0.002	17.2± 0.15	0.48± 0.001	23.1± 0.13	0.53± 0.002	31± 0.11	0.94± 0.003	34.45± 0.101
48	0.183± 0.001	12.4± 0.20	0.3± 0.003	***16± 0.15**	0.3± 0.003	***18± 0.17**	0.59± 0.002	25.3± 0.11	0.65± 0.001	***35± 0.21**	1.01± 0.003	***37± 0.26**
60	0.112± 0.001	12± 0.15	0.27± 0.001	14± 0.59	0.3± 0.001	16.8± 0.11	0.64± 0.001	28.3± 0.13	0.73± 0.002	33.2± 0.18	0.92± 0.003	34.91± 0.17
72	0.083± 0.001	11.4± 0.23	0.22± 0.001	10± 0.15	0.2± 0.003	15.3± 0.11	0.76± 0.003	***32± 0.11**	0.81± 0.003	29± 0.23	0.86± 0.02	31.23± 0.23
84	0.052± 0.001	10± 0.15	0.21± 0.001	9.8± 0.11	0.2± 0.001	13.46± 0.13	0.68± 0.002	20.7± 0.17	0.77± 0.002	25.3± 0.18	0.79± 0.002	27.56± 0.15
96	0.033± 0.002	10.3± 0.12	0.19± 0.003	9.4± 0.026	0.2± 0.001	10.73± 0.17	0.6± 0.003	16.5± 0.11	0.69± 0.003	21.02± 0.32	0.72± 0.003	24.56± 0.20

Experiments were conducted in triplicate and results were the average of these three independent trials.* represents the significant value ($p \leq 0.05$)

3.7 Discussion

Lipase production from different lactic acid bacteria like *L. lactis*, *L. brevis* and *L. plantarum* with various readily availablenon-edible oils like camphor oil, peppermint oil, rose oil, eucalyptus oil, lemon oil and olive oil as substrate was carried out. From Table 3.1 and Figure 3.1 it was evident that the most suitable substrate for lipase production was olive oil as very good growth of organism was observed.

It is evident from figure 3.2 that the maximum lipase activity of 27 U/ml was observed with 48 hrs of incubation for the co-culture of *L. brevis* + *L. plantarum* whereas *L. lactis* + *L. brevis* and *L. lactis* + *L. plantarum* showed maximum activity of 23.42 U/ml and 23 U/ml respectively at 72 hrs. Production of lipase activity increased with the time of fermentation. However, the increase in activity dropped after certain incubation period which can be attributed to many reasons. As this enzyme production seems to be growth dependent, exhaustion of nutrients can be one possible reason for the decrease in activity. Another reason can be accumulation of bacterial toxins.

Figure 3.3 shows that maximum lipase activity of 31 U/ml was observed for co-culture of *L. plantarum* + *L. brevis* at pH 5.0 and for co-cultures of *L. lactis* + *L. brevis* and *L. lactis* + *L. plantarum* it was 30 U/ml and 26.35 U/ml respectively at pH 5.5. As the metabolic activities of the microorganisms are very much sensitive to the pH change, lipase production also is found to be affected. The enzyme activity decreases if the pH level is higher or lower than the optimum value [156].

From Figure 3.4 it is clear that 30 ^{o}C is the optimum temperature for lipase production in *L. brevis* + *L. plantarum* (24 U/ml) and 35 ^{o}C for *L. lactis* + *L. brevis* and *L. lactis* + *L. plantarum* (24 U/ml and 23 U/ml) respectively. Higher temperature is found to have some

adverse effect on the metabolic activities of the microorganism and it is also reported by various scientists that the metabolic activities of the microorganism become slow at lower temperature [157-158].

Importance of inoculum concentration for microbial fermentation processes is widely known. From Figure 3.5, it was found that 2 ml of inoculum volume was the optimum required for maximum lipase activity of 22.35 U/ml, 22 U/ml and 23.28 U/ml for *L. lactis* + *L. brevis*, *L. lactis* + *L. plantarum* and *L. brevis* + *L. plantarum* respectively.

From Figure 3.6 it was observed that the maximum enzyme activity of 24.35 U/ml, 27 U/ml and 28.2 U/ml was obtained with 2 ml of substrate volume for *L. lactis* + *L. brevis*, *L. lactis* + *L. plantarum* and *L. brevis* + *L. plantarum* respectively. Hence all further experiments in this study were done using olive oil as substrate.

The total protein concentration was found to be 3.5mg/ml. Maximum enzyme activity of 32 U/ml from co-culture of *L. lactis* and *L. brevis* was obtained at pH 5.5, temperature 35 ^{o}C, incubation period of 72 hrs, 2 ml of inoculum volume (i.e. 1ml of *L. lactis* + 1ml of *L. brevis*) and 2 ml of substrate volume. The activity decreased with increase in the values of the parameters. Similarly the activity of lipase enzyme from co-culture of *L. lactis* and *L. plantarum* was 35 U/ml obtained with the same above parameters. Highest enzyme activity of 37 U/ml was obtained from co-culture of *L. brevis* and *L. plantarum* at pH 5, temperature 30 ^{o}C, 48 hrs of incubation period with 2 ml of inoculum volume (where the number of cells inoculated for *L. brevis* was 1.04×10^{6} cells/ml and 1.20×10^{6} cells/ml for *L. plantarum*) and 2 ml of substrate volume. It was also observed that when compared to the enzyme activity of single cultures of 13 U/ml for *L. lactis*, 16 U/ml for *L. brevis* and 18 U/ml for *L. plantarum*, the enzyme activity of co-

cultures were more [159]. The cell dry weight determination showed similar results which was evident from Table 3.2.

3.8 Optimization of physical parameters using statistical tools

Culture conditions like temperature, pH, incubation period, substrate volume and inoculum volume play a very important role in fermentation process. Improving fermentation conditions is the most frequently used operation in biotechnology to obtain maximum cell density, high yields of the desired metabolic product, or enzyme levels in the microbial system. By manipulation of growth environment one can elicit enhanced production in microorganisms.

The conventional practice of single factor optimization does not depict the combined effect of all the factors involved. This process is time consuming, and also ignores the combined interactions between physicochemical parameters. The limitation of single factor optimization can be eliminated by statistical design techniques. The application of such techniques in fermentation process development can result in improved yields, reduced process variability, closer confirmation of the output response (product yield) to nominal and target requirements, reduced development time and overall costs.

3.8.1 Response surface methodology

Conventional single factor optimization method requires number of experiments to determine the optimum levels and does not represent the combined effect of all the parameters involved. These limitations of single parameter optimization can be eliminated by optimizing all the affecting parameters collectively with statistical design techniques using Response Surface Method (RSM) [160]. It is a collection of mathematical and statistical techniques to optimize a response (output variable) which is influenced by several independent variables (input variables). The application of RSM to design optimization is aimed at reducing the cost of expensive

analysis methods (e.g. finite element method or Computational Fluid Dynamics (CFD) analysis) and their associated numerical noise. In recent years, this approach has become a very popular tool for optimization of various process parameters. But RSM too has some major drawbacks like, i) it is usually quadratic, thus being incapable to be applied in complicated nonlinear cases; ii) the co linearity problems between factors may exist; and iii) sensitivity analysis of input variables is difficult to perform because of the presence of cross interactions. These drawbacks of RSM can be removed by Artificial Neural Network (ANN).

3.8.2 Artificial neural network

An ANN is a highly interconnected network consisting of many simple processing elements capable of performing a massively parallel computation for data processing inspired by the elementary principles of the nervous system. An ANN imposes no restrictions on the type of relationship governing the dependence of the growth parameters on the various running conditions. ANN based simulators can also be used to generate response surfaces and has shown better predictability than other multivariate algorithm in many instances.

RSM was employed to optimize media components [160] for extracellular lipase production by *Fusarium solani* strain SKWF7 isolated from dairy wastewater. Maximum lipase production of 73.3 U/ml was reported where 1.7-fold increase was observed with RSM-guided design of experiments compared to unoptimized medium. RSM has been used for optimization of physico-chemical parameters, viz. temperature, oil concentration, inoculum volume, pH and incubation period on lipase production from *Enterobacter aerogenes* resulting in 1.4-fold increase in lipase activity. The optimum levels of parameters were found to be 34 ^{o}C, oil concentration of 3%, inoculum volume of 7%, pH 7 and incubation time 60 hrs [161].

RSM was employed on five different variables that influence lipase production from *Ganoderma lucidum* [162]. Maximum lipase yield of 4838 U/gds was obtained with canola oil cake (12.50 g), moisture level (60%), pH (4.5), olive oil as inducer (2.0%) and incubation period (96 hrs) at 30 ^{o}C. Various fermentation parameters were optimized using Placket-Burmen design and central composite design methods for cold active lipase production from *Moritella* sp, a psychrophilic bacterium. 30.56 U/ml of production was obtained after using optimized parameters [163].

There is report on ANN based computations being applied for developing predictive models as a result of combined effects of three environmental factors (i.e. temperature, pH and water activity) on thermal inactivation rate of *Escherichia coli*. In this paper, the modeling ability of ANN algorithm compared to that of RSM method showed the superiority of the accurate prediction by ANN-based approach [164]. The effect of cultural parameters, were studied using RSM and ANN for production of thermostable lipase from thermophilic *Geobacillus* sp. strain ARM [165].

RSM and ANN were used for optimization of culture parameters for the lipase production of a newly isolated *Staphylococcus xylosus* where a 3.5 fold increase in production was obtained with optimized parameters. These results demonstrated a high predictive accuracy of artificial neural network compared to response surface methodology. The lipase was also found to be acidic and alkaline resistant [166].

From the above reports, it is evident that RSM is a much better technique than single parameter optimization involving factorial design and regression analysis, which helps in evaluating the effective factors and building models to study the interaction and select optimum conditions of variables for a desirable response in microbial enzyme production. But ANN is

superior and more accurate modeling technique when compared to RSM method as it represents the nonlinearities in a much better way. However, response surfaces plotted by 3-D plots can provide a good way for visualizing the parameter interaction. Therefore, now-a-days both techniques are used in unison for predicting optimum fermentation process conditions for microbial enzyme production.

3.8.3 Genetic algorithm

Genetic algorithm (GA) is a stochastic optimization technique that searches for an optimal value of a complex objective function and are used to solve complicated optimization problems by simulation or mimicking a natural evolution process [167]. GA has been successfully used as a tool in computer programming, artificial intelligence, optimization, and neural network training and information technology. The major advantage of genetic algorithms over other conventional optimization techniques is the flexibility it provides in giving the objective function and constrains [77].

A comprehensive GA technique for predicting an optimum arbitrary feed-in profile has been recently reported [168]. A common feature of almost all the optimization techniques is to control feed-in, feed-out and recycling rate. But genetic algorithm being so flexible tool for optimization, it is quite possible to seek for optimum values of other reaction parameters also, e.g., reaction time, amount of initial inoculum, initial substrate concentration etc. In this thesis emphasis has been given to see that these parameters are also playing important role to control the reaction dynamics.

Garlapati, et. al. [169] used GA and Particle Swarm Optimization (PSO) approaches for optimization of fermentation conditions of lipase production for enhanced lipase activity and

found that PSO approach has slightly better performance and possesses better convergence and computational efficiency than the GA approach.

GA has been used for optimization of production parameters for protease production from newly isolated *Pseudomonas* sp. RAJR 044 in a bubble column bioreactor operated in fed-batch mode [170]. Increase in production was observed with optimized values. GA was found to be minimizing reaction time and maximizing product concentration.

ANN and GA techniques were used to optimize process parameters that enhanced lipase production in soil organisms. 7.69 U/ml of lipolytic activity was obtained using the ANN-GA method [171]. Development of high speed computing capability now paves the way for using complex and computationally intensive algorithms for on line process optimization.

3.8.4 Taguchi method

The levels of the factors affecting the enzyme yield were optimized via the Taguchi method which was proposed by "Genichi Taguchi". This method is now widely used in different fields like biotechnology, engineering, marketing [172]. The method utilizes a fractional factorial design in form of an orthogonal array containing a representative set of all possible combinations of experimental conditions. Multi enzyme scouring process optimization was done using statistical tool Taguchi [173]. Scaling and adjustment factors were optimized using this method.

3.8.5 Support Vector Machines

Support Vector Machines (SVMs) is most important method of supervised learning, which analyses and recognizes data patterns, useful for classification and regression. The advantage with this type of algorithm is attaining the global minimum, and avoids the local minimum as in other methods such as neural networks. Cai et. al. classified enzymes by support vector machines into functional families [174]. Statistical optimization of growth parameters

was done using SVR for amylase production from *Streptomyces lonarensis* strain NCL 716 [175]. An enzyme activity of 297 U/ml was obtained using these optimization methods.

3.9 Statistical optimization of culture parameters for lipase production from *Lactococcus lactis* - Response surface methodology with Genetic algorithm (GA)

RSM had not only been used for optimization of culture parameters in the fermentation process [176-177] but also for studying the combined effects of medium components. The principle of RSM was described by Khuri and Cornell [178]. Using RSM, the relationship among the variables, i.e. initial pH, temperature, incubation period, inoculum volume and substrate volume were expressed mathematically in the form of a polynomial model, which gave the response as a function of relevant variables. A functional relationship between response and independent process parameters based on a full quadratic model is postulated by:

$$y_i = a_0 + \sum_{i=1}^{5} a_i x_i + \sum_{i=1}^{5}\sum_{j=1}^{5} a_{ij} x_i x_j$$

Where$y_i$was the predicted response (lipase production) used as a dependent variable; x_i (i = 1, 2, 3, 4 and 5) were the input predictors or controlling variables; and a_0, a_i (i = 1, 2, 3, 4, 5) and a_{ij}(i = 1, 2, 3, 4, 5; $j = i, \ldots, 5$) were the model coefficient parameters. The coefficient parameters were estimated by multiple linear regression analysis using the least-squares method. Designs of this type are usually chosen when there is suspecting curvature in the response surface. These process parameters and their different levels are presented in Table 3.3.

Table 3.3: Process parameters and levels of the experiment

Level	pH	Temperature (^{o}C)	Incubation Period (hours)	Inoculum volume(ml)	Substrate volume(ml)
1	5	25	12	0.5	0.5
2	6	30	24	1	1
3	7	35	36	1.5	1.5
4	8	40	48	2	2
5	-	-	60	-	-

Thus a total of 4*4*5*4*4 = 1280 experiments were conducted to formulate RSM model for lipase production using the pH, Temperature (^{o}C), Incubation period (hrs), Inoculum volume (ml) and Substrate volume (ml).The analysis of the developed RSM models is shown in Table 3.4.

Table 3.4: Response Surface Regression: Yield versus pH (pH), Temperature (T), Incubation period (IP), Substrate (S) and Inoculum volume (I).

Estimated Regression Coefficients for yield

Term	Coef	SE Coef	T	P
Constant	-2.06068	0.066417	-31.026	0.000
Ph	0.43559	0.012695	34.312	0.000
T	0.03587	0.002539	14.129	0.000
Ip	0.00570	0.000529	10.780	0.000
S	0.07126	0.016449	4.332	0.000
I	0.01294	0.016449	0.787	0.432
ph*ph	-0.03518	0.000879	-40.011	0.000
t*t	-0.00062	0.000035	-17.683	0.000
ip*ip	-0.00007	0.000004	-18.331	0.000
s*s	-0.01948	0.003517	-5.540	0.000
i*i	-0.00482	0.003517	-1.369	0.171

ph*t	0.00146	0.000141	10.398	0.000
ph*ip	-0.00000	0.000046	-0.047	0.963
ph*s	-0.00194	0.001407	-1.377	0.169
ph*i	0.00001	0.001407	0.007	0.994
t*ip	-0.00002	0.000009	-1.725	0.085
t*s	-0.00019	0.000281	-0.691	0.490
t*i	0.00003	0.000281	0.122	0.903
ip*s	0.00001	0.000093	0.100	0.921
ip*i	0.00002	0.000093	0.266	0.791
s*i	0.00005	0.002814	0.019	0.985

Where Coef- coefficient, T-T test, SE coef- standard coefficient, P-probability

S = 0.0314592 PRESS = 1.27980 R-Sq = 76.27% R-Sq (pred) = 75.63% R-Sq (adj) = 75.89%

Response Surface Regression: Yield versus pH (pH), Temperature (T), Incubation period (IP), substrate (S) and Inoculum volume (I).

The analysis of variance (ANOVA) and the F-ratio test have been performed to justify the goodness of fit for this RSM model. Both the high adjusted-R^2 value and the close to zero *p*-value in the analysis of variance (ANOVA) presented in Table 3.5 show that this RSM model for lipase production has a satisfactory goodness of fit.

Table 3.5: Analysis of variance for yield

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	20	4.00493	4.00493	0.20025	202.34	0.000
Linear	5	1.63394	1.32525	0.26505	267.81	0.000
Ph	1	0.86734	1.16518	1.16518	1177.33	0.000
T	1	0.69829	0.19757	0.19757	199.63	0.000
Ip	1	0.05813	0.11502	0.11502	116.22	0.00
S	1	0.00651	0.01857	0.01857	18.77	0.000
I	1	0.00367	0.00061	0.00061	0.62	0.432
Square	5	2.25860	2.25860	0.45172	456.43	0.000
ph*ph	1	1.58435	1.58435	1.58435	1600.88	0.000

t*t	1	0.30948	0.30948	0.30948	312.70	0.000
ip*ip	1	0.33254	0.33254	0.33254	336.01	0.000
s*s	1	0.03037	0.03037	0.03037	30.69	0.000
i*i	1	0.00186	0.00186	0.00186	1.87	0.171
Interaction	10	0.11239	0.11239	0.01124	11.36	0.000
ph*t	1	0.10700	0.10700	0.10700	108.12	0.000
ph*ip	1	0.00000	0.00000	0.00000	0.00	0.963
ph*s	1	0.00188	0.00188	0.00188	1.90	0.169
ph*i	1	0.00000	0.00000	0.00000	0.00	0.994
t*ip	1	0.00295	0.00295	0.00295	2.98	0.085
t*s	1	0.00047	0.00047	0.00047	0.48	0.490
t*i	1	0.00001	0.00001	0.00001	0.01	0.903
ip*s	1	0.00001	0.00001	0.00001	0.01	0.921
ip*i	1	0.00007	0.00007	0.00007	0.07	0.791
s*i	1	0.00000	0.00000	0.00000	0.00	0.985

Where DF- Degrees of freedom, ADJ SS- adjusted sum of square, ADJ MS- adjusted mean square, F-ratio of variance within and between treatment, P- Probability, Residual Error 1259 1.24600, Seq SS - sequence of sum of squares

Total-1279 5.25094

3.9.1 Artificial Neural Network (ANN) with genetic algorithm (GA) for modeling and simulation

ANN was applied here to provide a nonlinear mapping between input variables (pH, temperature, incubation period, substrate volume and inoculum volume) and the output variable (enzyme production). The only disadvantage of ANN in comparison to RSM is the difficulty in explaining the relation between independent and dependent variables because of use of ambiguously defined weights, which is a black box.

In this investigation, the input variables of ANN include pH, temperature, incubation period (IP), substrate volume, inoculum volume, while the output variable is lipase yield. Hence,

a feed forward network trained with the back propagation algorithm was developed as shown in Figure 3.7

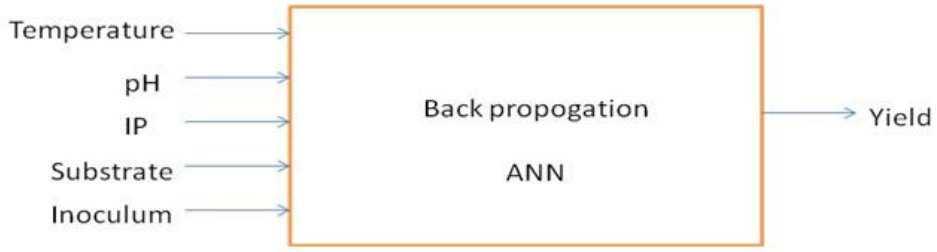


Figure 3.7: Feed forward network trained with the back propagation

Before training the network, the input and output datasets have been normalized within the range of 0.05 – 0.95 to prevent a specific factor from dominating the learning for the ANN model. The main reason for normalizing the data matrix where the variables have been measured in different units is to recast them into the dimensionless units to remove the arbitrary effect of similarity between the objects. Thus, using equation (1), the experimental data was normalized to make the neural network training more efficient prior to the use of the datasets.

$$x_n = 0.05 + 0.90*(x - x_{min})/(x_{max} - x_{min}) \quad (1)$$

where, x_{min} and x_{max} are the minimum and maximum values of x and x_n is the normalized data of the corresponding x. Once the best trained network is found, all the transformed data returns to their original value using equation (2)

$$x = x_{min} + (x_n - 0.05)*(x_{max} - x_{min})/0.9 \quad (2)$$

The value of mean square error (MSE) is used to check the ability of a particular architecture. It is observed that the mean square error of network decreases to the minimum value when the number of neurons is 21, which indicates that a network with 21 neurons in hidden layer can exhibit the best performance. In addition, based on trial and error procedure, for a double hidden layer ANN, the number of neurons is found to be 13 and 14 respectively for the best predictions. Figures 3.8 and 3.9 showed these two ANN architectures used in this study.

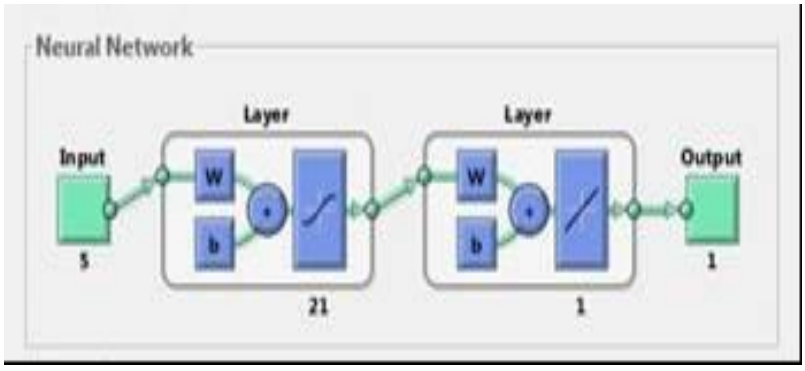


Figure 3.8: Architecture of a single intermediate layer ANN (5-21-1)

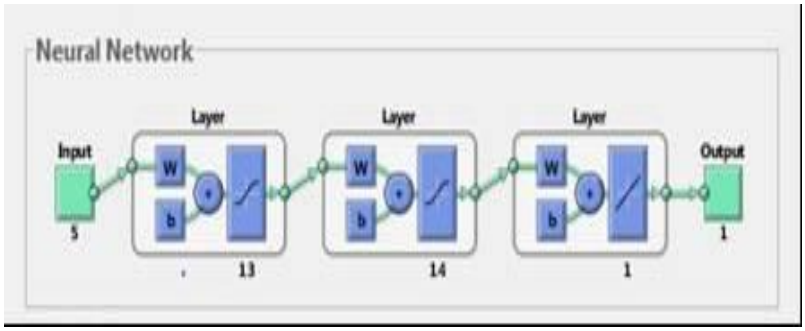


Figure 3.9: Architecture of a double intermediate layer ANN (5-13-14-1)

Following the training-testing-validation procedure and tenfold cross-validation recommended, 90% of the data was used for training and 10% of the data for testing. The training data was used to train the network with Levenberg–Marquardt function (trainlm). The neural network toolbox of MATLAB software package is used for training and testing the given data.

3.9.2 Results

The correlation coefficient (R) is a commonly used statistic and provides information on the strength of linear relationship between experimental and predicted values. For perfect prediction, all the data points should lie on the line inclined at 45^{o} from horizontal.The correlation for training and testing data 5-21-1 of ANN were presented in Figures 3.10 & 3.11.

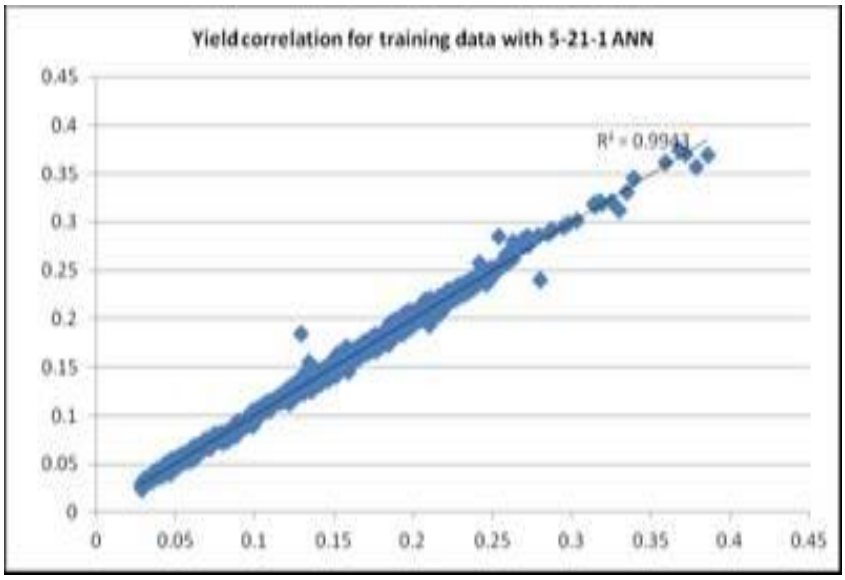


Figure 3.10: ANN showing correlation for training data 5-21-1

For ANN with architecture 5-21-1, Figure 3.9, showed the correlation coefficient to be 0.9943 for the training dataset and similarly Figure 3.10, showed the correlation coefficient to be 0.9953 for the testing dataset, indicating a very good correlation between the experimental and the predicted values.

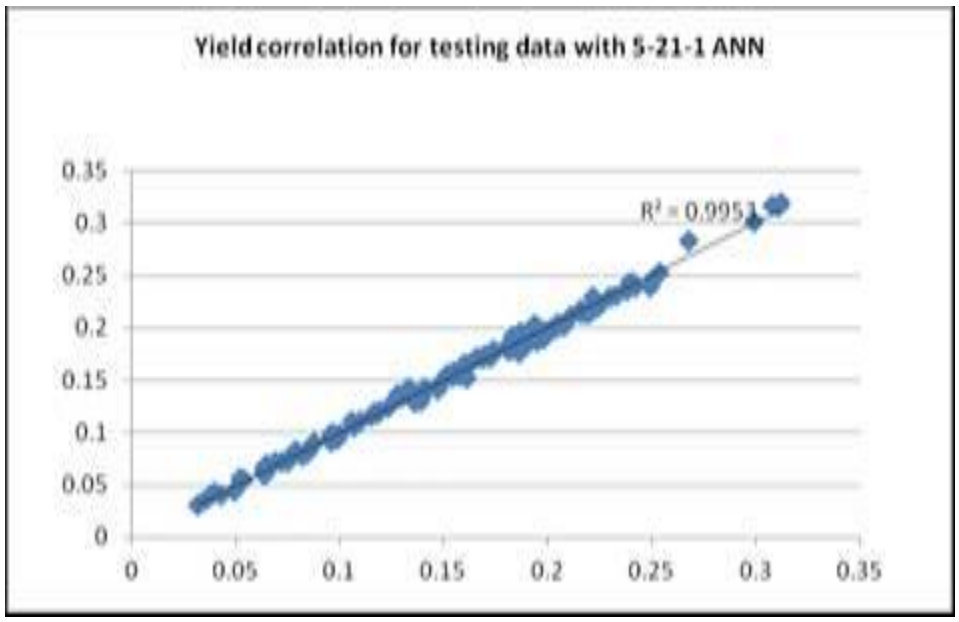


Figure 3.11: ANN showing correlation for testing data 5-21-1

Similarly, for ANN with architecture 5-13-14-1, Figure 3.12 showed the correlation coefficient to be 0.9967 for the training dataset and Figure 3.13 showed the correlation coefficient to be 0.9944 for the testing dataset, indicating a very good correlation between experimental and predicted values.

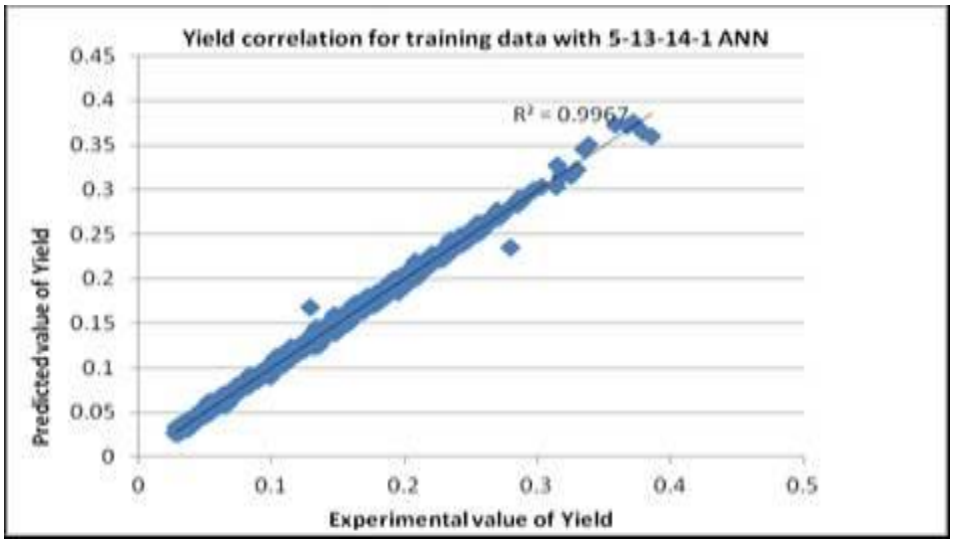


Figure 3.12: ANN showing correlation for training data 5-13-14-1

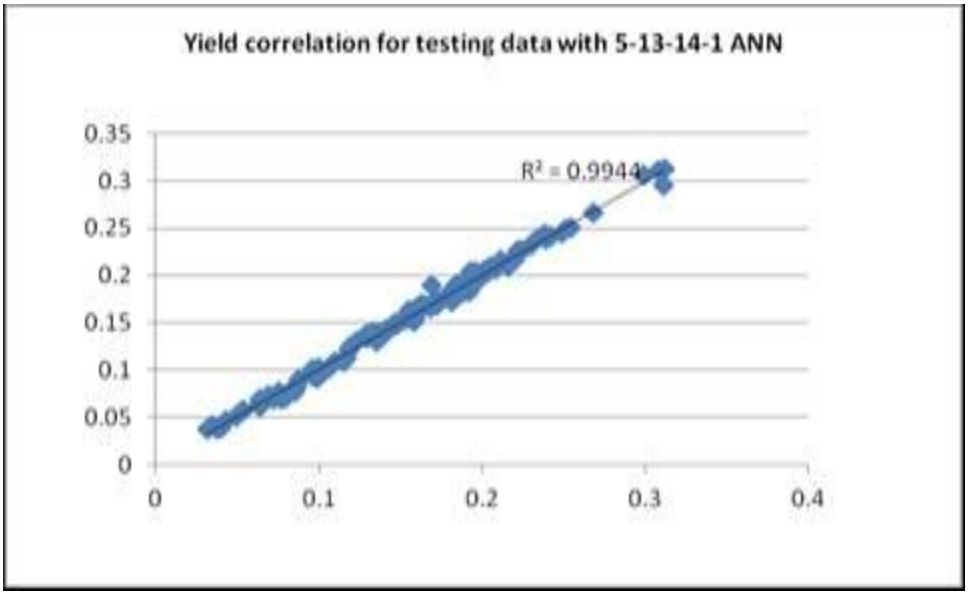


Figure 3.13: ANN showing correlation for testing data 5-13-14-1

Using RSM, ANN (5-21-1) and ANN (5-13-14-1), the genetic algorithm was applied to find out the parameter settings for the optimum yield of lipase, which is presented in Table 3.6

Table 3.6: Parameter setting for optimum yield of lipase

Statistical Optimization Tool	Optimum Parameters					Predicted yield	Experimental Yield	Specific activity
	pH	T	I.P	I	S			
ANN(5-13-14-1)	6.17	35.11	43.05	1.57	1.99	0.4083	0.4063	13 U/ml
ANN(5-21-1)	6.15	35.34	47.45	1.99	0.53	0.3842	0.3816	9 U/ml
RSM	6.90	36.31	38.58	1.32	1.58	0.2622	0.2643	8.4 U/ml

Where pH-pH, I.P-Incubation period, S-substrate, T-Temperature, I-Inoculum volume

The optimum operating conditions obtained from the quadratic form of the RSM and ANN models with GA were pH 6.7, temperature 35 ^{o}C, and inoculum volume of 1.5, substrate volume of 2 ml, with 13 U/ml of predicted lipase activity within 43 hrs of incubation. Figure 3.14 showed the graphical representation of predicted or optimum versus experimental yield for RSM, ANN (5-21-1) and ANN (5-13-14-1).

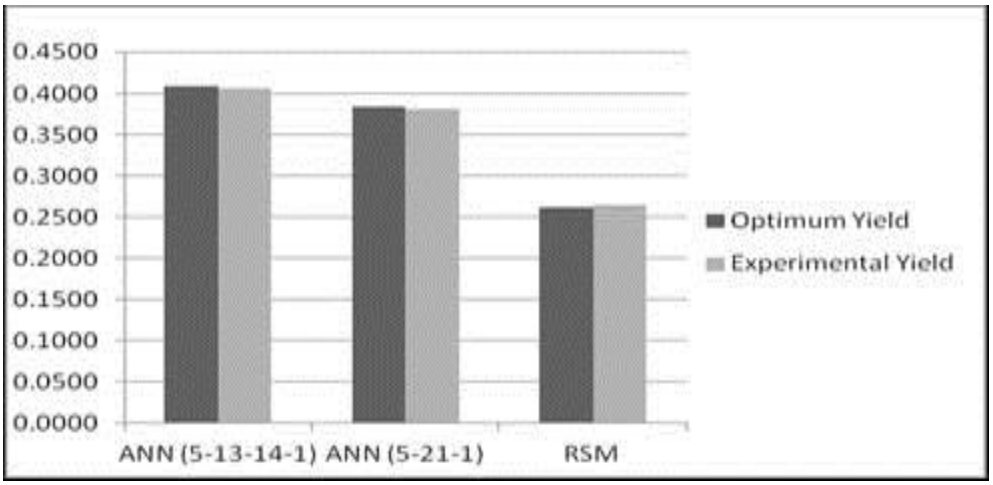


Figure 3.14: Predicted versus Experimental yield for RSM, ANN (5-21-1) and ANN (5-13-14-1)

Similarly, Figure 3.15 showed the specific activity of lipase for the three models developed.

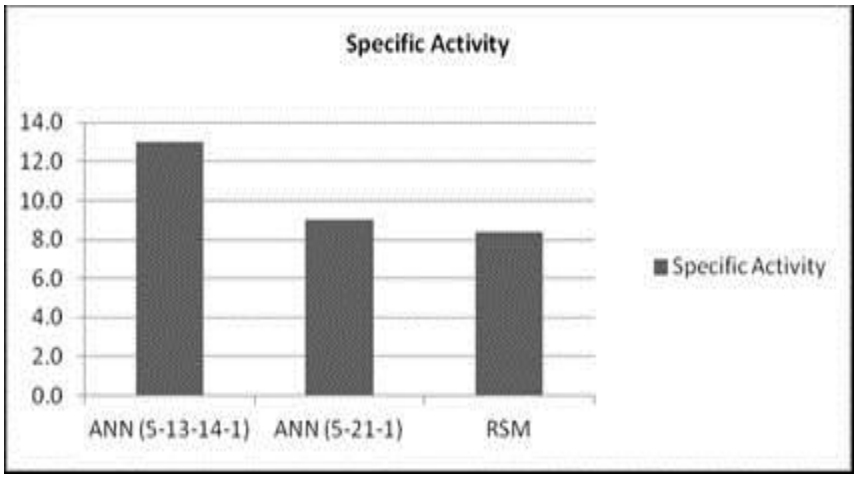


Figure 3.15: Specific activities (U/ml) of lipase obtained for RSM, ANN (5-21-1) and ANN (5-13-14-1) models

Thus, both the models based on ANN with GA performed much better than RSM with GA model and offered stable responses in predicting the combined interactions of the five independent variables, i.e. pH, temperature, inoculum volume, substrate volume, and incubation period with respect to extracellular lipase production [179]. The two intermediate layered ANN (5-13-14-1) with GA based approach was found to be the best for the lipase production.

3.9.3 Discussion

This study compared the performance of RSM with GA and ANN with GA in estimating the fermentation parameters (pH, temperature, inoculum volume, substrate volume and incubation period) for extracellular lipase production from *L. lactis.* There was no report till date on optimization of culture parameters for lipase production using this organism. Though both RSM with GA and ANN with GA models provided good quality predictions in this study, yet the ANN with GA showed a clear superiority over RSM with GA for both data fitting and estimation capabilities. Among the above two ANN models used, the two intermediate layered ANN (5-13-14-1) with GA based approach was found to be the best for the lipase production. The results

from these experiments could contribute to the development and use of this system on an industrial scale.

3.10 Statistical modeling and optimization of lipase production from co-cultures of *Lactococcus lactis* + *Lactobacillus plantarum* and *Lactococcus lactis* + *Lactobacillus brevis* using ANN and SVM with GA

The lipase production was first modeled with Artificial Neural Network (ANN) and Support Vector Machine (SVM) and optimized using Genetic Algorithm (GA) taking various physical parameters (pH, temperature, incubation period, inoculum and substrate volume) into account.

3.10.1 Artificial neural network

In this study, back propagation algorithm was used for training the data. The activation function and the number of neurons in each layer used for the training of neural network were collectively called as the neural network architecture. The selection of Neural Network architecture is one of the great challenges in the modelling of Neural Network. The Differential Evolution algorithm (DE) was used for training the networks and for the tuning of weights so as to determine the optimal architecture of neural networks.

3.10.2 Support vector machine model

Support Vector Machines (SVMs) is the most important method of supervised learning, which analyses and recognizes data patterns, useful for classification and regression. LS-SVM (least square-support vector machines) toolbox built in MATLAB environment was applied to the data obtained from the experiments. The parameters λ and ε were determined using the DE algorithm. The kernel function used is the radial basis function which is used extensively in literature.

3.10.3 Optimization of Lipase production using GA

The genetic algorithm is one of the popular methods for optimization of the objectives which are complex to solve. In a GA, a population of candidate solutions (called chromosomes) to an optimization problem was evolved towards fitter solutions in an iterative process. Each candidate solution was mutated and altered; traditionally, solutions were represented in binary as strings of 0s and 1s, but other encodings were also possible. The selection of chromosomes for the next generation was called reproduction, which was determined by the fitness of an individual. Different selection procedures were used in GA depending on the fitness values, of which proportional selection, tournament selection and ranking were the most popular procedures.

3.10.4 Results

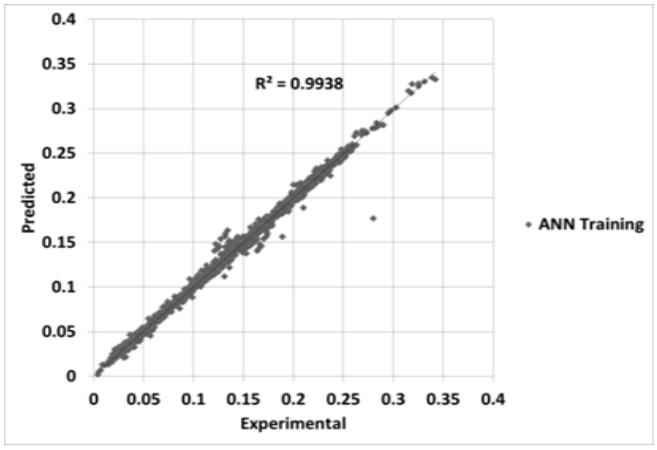
The ANN and SVM models for the lipase production were trained as explained in the algorithm above. The correlation coefficient is one of the statistical measures used to judge the goodness of fit of a model [180]. The correlation coefficient between two variables *X* and *Y* was measured using the Pearson product-moment coefficient, which takes the value between -1 and +1 inclusive. It is defined by the formula:

$$r = \frac{\sum_{i=1}^{n}(X_i-\bar{X})(Y_i-\bar{\bar{Y}})}{\sqrt{\sum_{i=1}^{n}(X_i-\bar{X})^2}\sqrt{\sum_{i=1}^{n}(Y_i-\bar{Y})^2}} \longrightarrow \text{Equation 1}$$

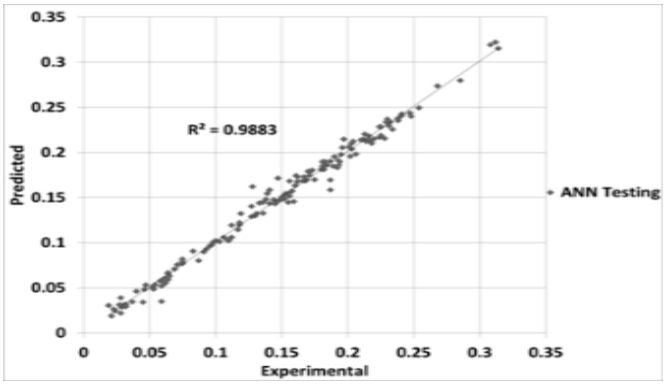
The X_i represents the original values as obtained in the experiments. $\overline{X}$is the

these original values. Similarly, *Y* represents the predicted values. The ideal prediction is supposed to give a value of *r* which is equal to one. Consequently, the ideal prediction leads to a straight line with slope 1, as the X-axis and Y-axis represent the experimental and predicted values by each of the methods employed. The complete data are divided into training, testing and

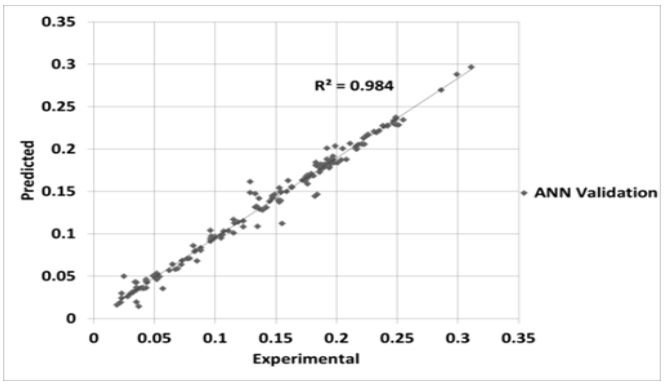
validation data (80%, 10% and 10% respectively). The training data were used for the training of the ANN and SVM models. The validation data was used to determine the optimal or the best ANN architecture or the best SVM parameters for good fit with the experimental results. The testing data was used to finally compare the ANN and SVM models based on their predictive capability at unknown process parameters. The correlation plots of training, testing and validating data of ANN and SVM for co-culture of *Lactococcus lactis and Lactobacillus plantarum* are shown in Figures 3.16 (a-c) and 3.17 (a-c) respectively.



(a)

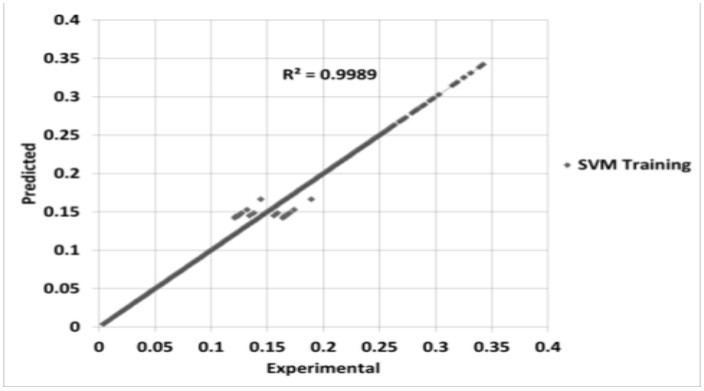


(b)

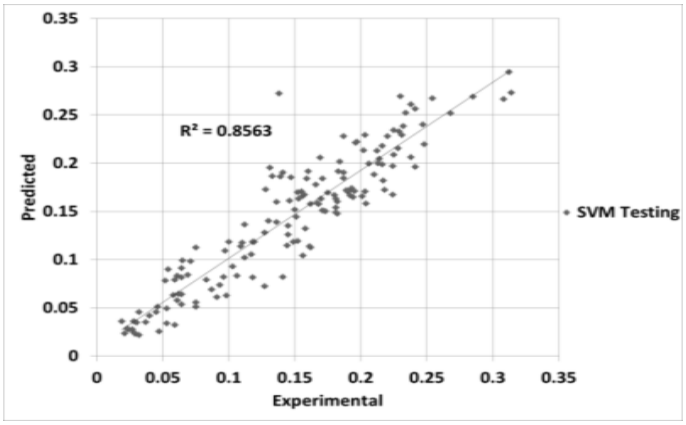


(c)

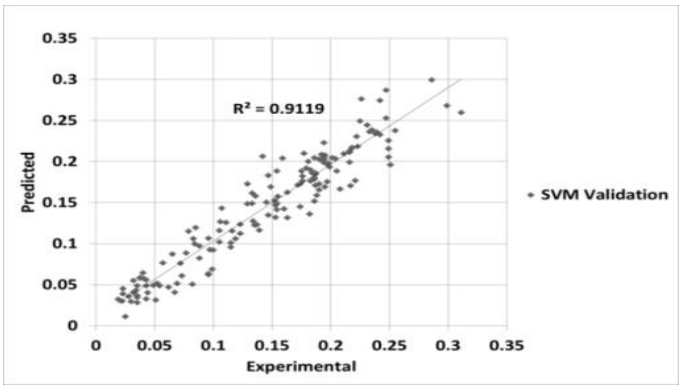
Figure 3.16: Plot of Predicted vs. Experimental (a) training data (b) testing data (c) validation data of ANN model for lipase production from co-cultures of *Lactococcus lactis* and *Lactobacillus plantarum*



(a)



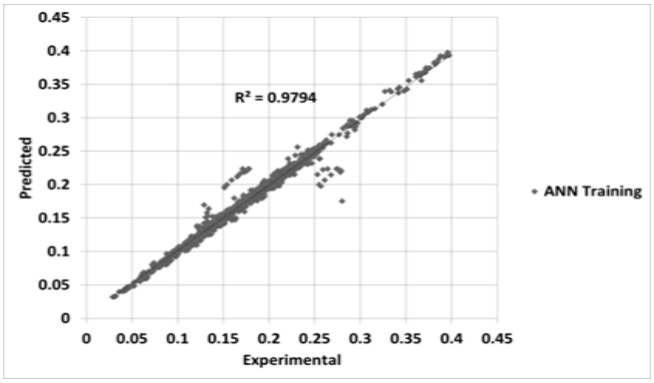
(b)



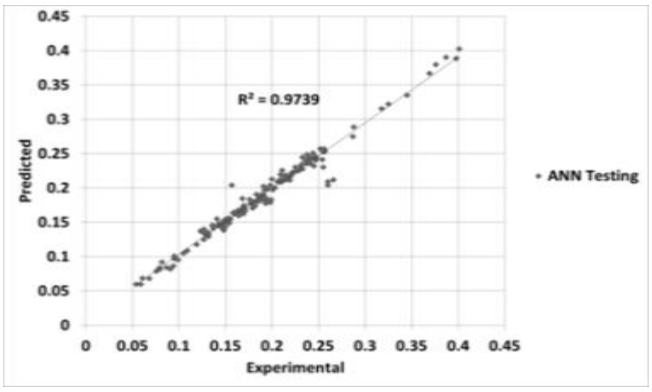
(c)

Figure 3.17: Plot of Predicted vs. Experimental (a) training data (b) testing data (c) validation data of SVM model for lipase production from co-cultures of *Lactococcus lactis* and *Lactobacillus plantarum*

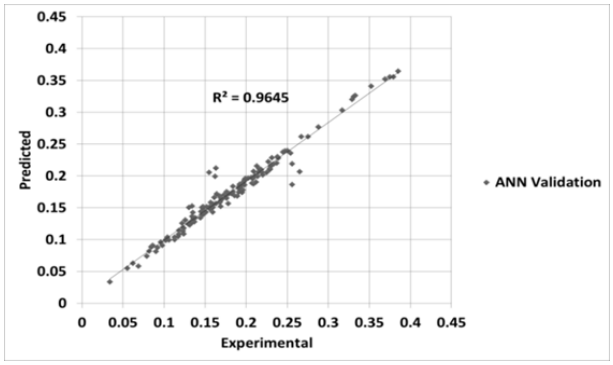
Similarly, the correlation plots of training, testing and validating data of ANN and SVM for co-culture of *L.lactis and L. brevis* are shown in Figures 3.18 (a-c) and 3.19 (a-c) respectively.



(a)

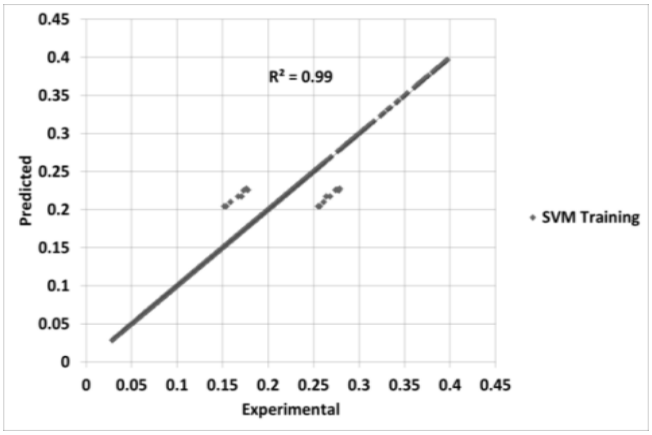


(b)

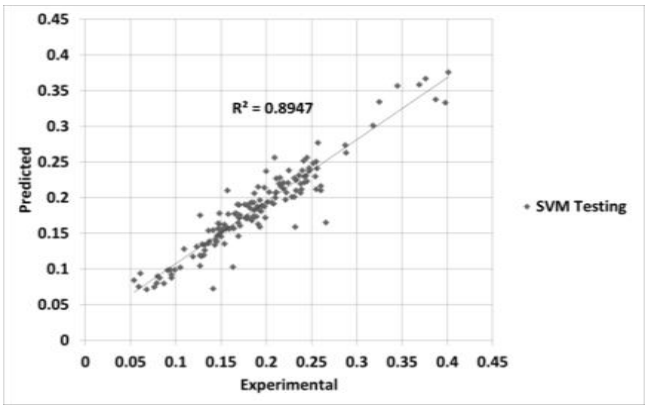


(c)

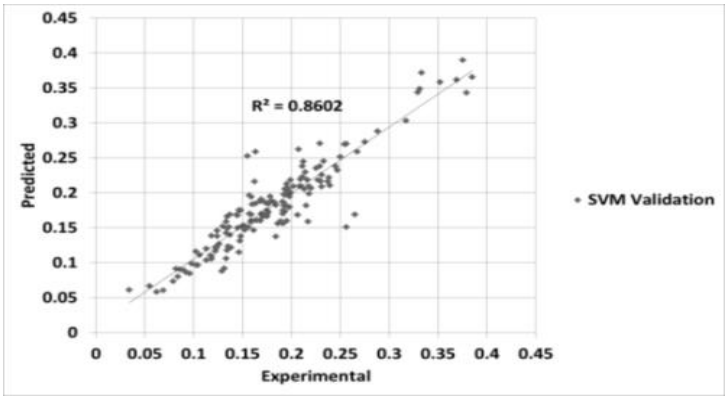
Figure 3.18: Plot of Predicted vs. Experimental (a) training data (b) testing data (c) validation data of ANN model for lipase production from co-cultures of *Lactococcus lactis* and *Lactobacillus brevis*



(a)



(b)



(c)

Figure 3.19: Plot of Predicted vs. Experimental (a) training data (b) testing data (c) validation data of SVM model for lipase production from co-cultures of *Lactococcus lactis* and *Lactobacillus brevis*

It is seen in general from these figures that SVM has good correlation with the experimental results for the training data set. Hence, it is seen that ANN has more predictability of lipase yield due its good correlation with validation and testing data. Later, the lipase production was optimized using the GA toolbox as mentioned in the previous sections. The optimal lipase yield as obtained from the GA for ANN and SVM models of lipase production are shown in Tables 3.7 and 3.8 respectively.

Table 3.7: Experimental and predicted yield of lipase obtained for co-culture of *L. lactis* and *L. plantarum*

Co-culture of *Lactococcus lactis* and *Lactobacillus plantarum*								
Model	**Input parameters**					**Experimental Yield**	**Predicted Yield**	**% deviation**
	pH	**Temp(^{o}C)**	**Incubation (hrs)**	**Inoculum volume (ml)**	**Substrate volume (ml)**			
SVM	5.492	34.828	76.230	1.768	1.760	0.414	0.428	3.365
ANN	5.450	34.944	77.213	0.518	2.000	0.400	0.412	2.887

Table 3.8: Experimental and predicted yield of lipase obtained for co-culture of *Lactococcus lactis* and *Lactobacillus brevis*

Co-culture of *Lactococcus lactis* and *Lactobacillus brevis*								
Model	**Input parameters**					**Experimental Yield**	**Predicted Yield**	**% deviation**
	pH	**Temp (^{o}C)**	**Incubation Period (hrs)**	**Inoculum volume (ml)**	**Substrate volume (ml)**			
SVM	5.658	25.019	72.980	1.486	1.997	0.350	0.361	3.288
ANN	5.574	29.934	60.295	1.695	1.799	0.362	0.369	1.869

The input parameters represent the process parameters using which the optimal yield was obtained. The predicted yield was the yield at optimum settings, obtained from the GA toolbox for the ANN and SVM models. The Experimental yield represented the experimental results obtained. The final column in tables 3.7 and 3.8 represented the deviation in the lipase yield from the experimental value found out by

$$\%\ deviation = \frac{|experimental\ yield - predicted\ yield|}{predicted\ yield} \times 100$$

The % deviation value is less than 5% in all the models obtained.

Further, it was observed that the *% deviation* from ANN model is lesser than the SVM model. This suggested that ANN is a better predictive model due to its lesser % deviation and better correlation with the experimental results. However, there are a few more considerations which have been identified in the literature. The computational time taken from ANN was 122.93 seconds, whereas that from SVM was 0.45 seconds. Thus, it was seen that SVM takes less computational time than ANN. Similar results were observed with the lipase production studies. Further, it was seen that the prediction of lipase production was significantly accurate. The % deviation from the predicted results was under 5%. Hence, it can be concluded that both ANN and SVM are having comparable results. The higher yield obtained from SVM model is justified due to the generalization capability. When a model is allowed to completely predict the results based on the present experimental results, there is a general tendency to incur more error at unknown data points. However, if the data is allowed, have little bit of error in the prediction with the present experimental data points, the tendency to overfit the data is less (this phenomenon is known as generalization capability as explained by Cristopher and Bishop). Hence, it can be seen that SVM is better than ANN is terms of computational time and

generalization capability. But ANN is better than SVM in terms of correlation coefficient and % deviation.

The lipase activity with classical method of optimization was 32 U/ml from co-culture of *L. lactis* and *L. brevis*. Similarly the activity of lipase enzyme from co-culture of *L. lactis* and *L. plantarum* was 35 U/ml whereas with above statistical approach the activity was found to be 33.5 U/ml from co-culture of *L. lactis* + *L. brevis* and 36 U/ml from *L. lactis* + *L. plantarum.*

3.10.5 Discussion

Considering the computational time and the generalization capability of both the models, it was found that SVM was better than the ANN model. Considering the % deviation and the correlation coefficient with the experimental data, it was found that ANN was better than SVM.

Further, it was also seen that on application of GA, the lipase production was optimized and the predicted results were validated by experiments with the input parameters obtained from GA for optimal yield.

3.11 Statistical optimization of lipase production from co-cultures of *Lactobacillus brevis* and *Lactobacillus plantarum* using ANN with GA and Taguchi method

3.11.1 Genetic Algorithm

In the above study Artificial Neural Network enhanced Genetic Algorithm (ANN-GA) and Bayesian Regularized Artificial Neural Network enhanced Genetic Algorithm (BR-ANN-GA) were explored in order to produce lipase economically from the co-culture of *L. brevis* and *L. plantarum*. Taguchi method had been applied to enhance the effectiveness of optimization.

3.11.2 Artificial Neural Network Model with GA

In the previous studies, it was found that feed forward back propagation neural networks are being used to train the neural network which is very reliable. However, the normal back-

propagation algorithm suffers the problem of not being able to give better generalization. The generalization is given better by using Bayesian regularization (BR-ANN). In this study, a comparison was made between models trained with back-propagation algorithm and also the back-propagation algorithm enhanced with Bayesian regularization.Genetic algorithm is a more flexible tool used here for reducing reaction time and increasing the product concentration.

3.11.3 Taguchi Design

The levels of the factors affecting the enzyme yield were optimized via the Taguchi method. The significance of fermentation parameters like pH, temperature, incubation period, substrate volume and inoculum volume was understood using this method. The method utilizes a fractional factorial design in form of an orthogonal array containing a representative set of all possible combinations of experimental conditions. The appropriate Taguchi orthogonal array for 5 parameters is L25. Table 3.9 represents the L25 array, the first column represents the experiment number and the subsequent columns represent the parameters and the rows represent the parameter settings in the different experiments. Taguchi recommended a logarithmic transformation of Mean Square Error (MSE) (Signal to noise ratio – S/N) in order to increase the robustness of design against noises and to accommodate a wide range of data for the analysis of the results. This method helps in proper analysis of results and consistency in the predicted performance. Table 3.9 also shows the S/N ratios for each parameter and the levels are found using the statistical tool Minitab v16.

Table 3.9: L25 array and S/N ratio values obtained by Taguchi method

pH	Temperature (^{o}C)	Incubation Period (hrs)	Inoculum (ml)	Substrate (ml)	Yield	S/N ratios
5	25	12	0.5	0.5	0.112	19.01564
5	30	24	1	1	0.316	10.00626
5	35	36	1.5	1.5	0.243	12.28787
5	40	48	2	2	0.087	21.20961
5	25	60	0.5	0.5	0.255	11.8692
5.5	25	24	1.5	2	0.153	16.30617
5.5	30	36	2	1	0.172	15.28943
5.5	35	48	1	0.5	0.308	10.22899
5.5	40	60	0.5	0.5	0.186	14.60974
5.5	30	12	1	1.5	0.134	17.4579
6	25	36	1.5	1	0.201	13.93608
6	30	48	0.5	1.5	0.19	14.42493
6	35	60	1	2	0.187	14.56317
6	40	12	1.5	1.5	0.181	14.84643
6	35	24	2	0.5	0.222	13.07294
6.5	25	48	1	2	0.115	18.78604
6.5	30	60	1.5	0.5	0.158	16.02686
6.5	35	12	2	1	0.189	14.47076
6.5	40	24	2	1.5	0.187	14.56317
6.5	40	36	0.5	2	0.206	13.72266
5	25	60	2	1.5	0.206	13.72266
5.5	30	12	1	2	0.132	17.58852
6	35	24	0.5	0.5	0.229	12.80329
6.5	40	36	1	0.5	0.209	13.59707
6.5	35	48	1.5	1	0.254	11.90333

3.11.4 Results

The ANN was trained with plane back-propagation network as well as with Bayesian Regularization. The correlation was one of the statistical measures used to judge the goodness of fit of a model. Correlation between two variables X and Y was measured using the Pearson product-moment coefficient, which takes the value between -1 and +1 inclusive which is defined by the equation 1 in setion 3.10.4.

The correlation plot of the ANN model for the 5 parameter is shown in Figure 3.20 and the architecture is shown in Figure 3.21

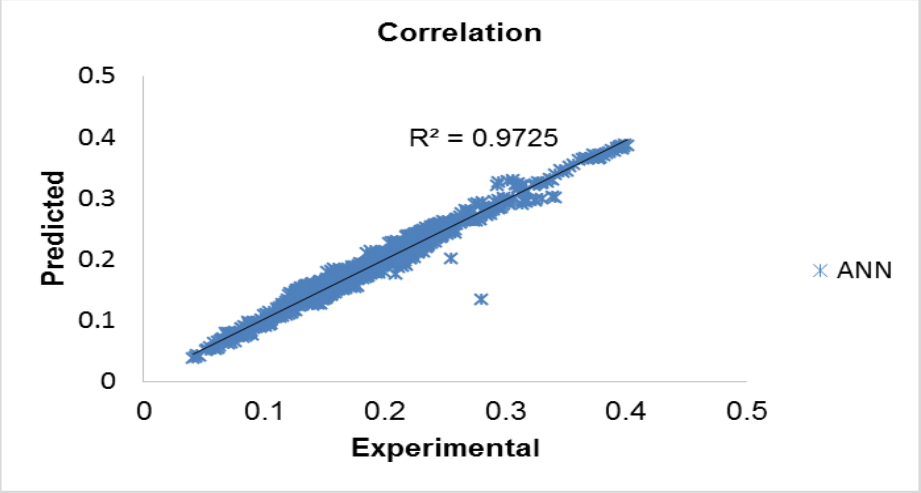


Figure 3.20: Correlation plot of ANN for 5- parameters

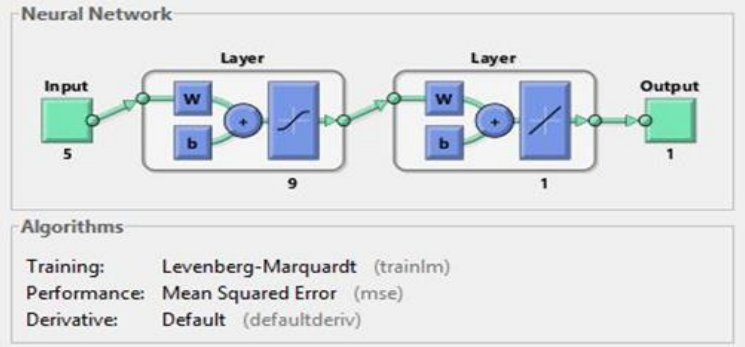


Figure 3.21: The architecture of ANN for 5-parameter

Similarly, the correlation plot for Bayesian regulation used in addition to back propagation was shown in Figure 3.22. The architecture of BR-ANN is shown in Figure 3.23

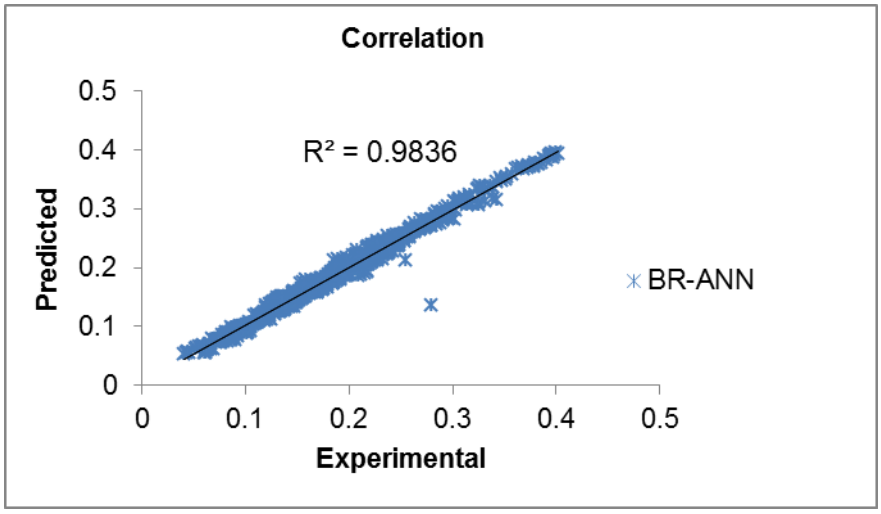


Figure 3.22: Correlation plot of BR-ANN for 5-parameters

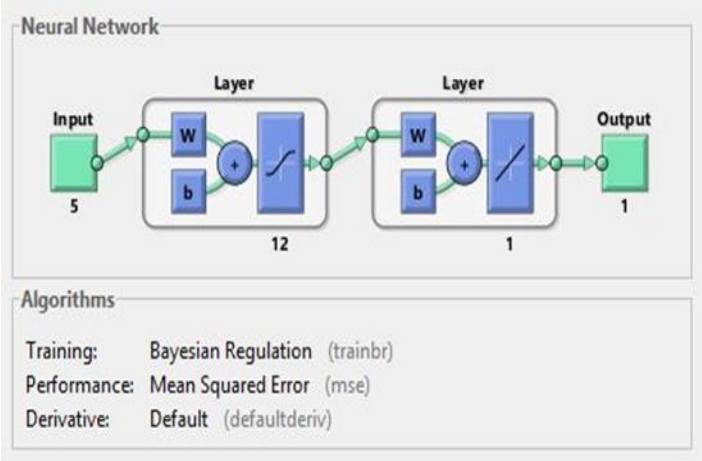


Figure 3.23: The architecture of BR-ANN for 5-parameter

The correlation plot of the ANN model for the 3 parameters is shown in Figure 3.24 and the architecture is shown in Figure 3.25.

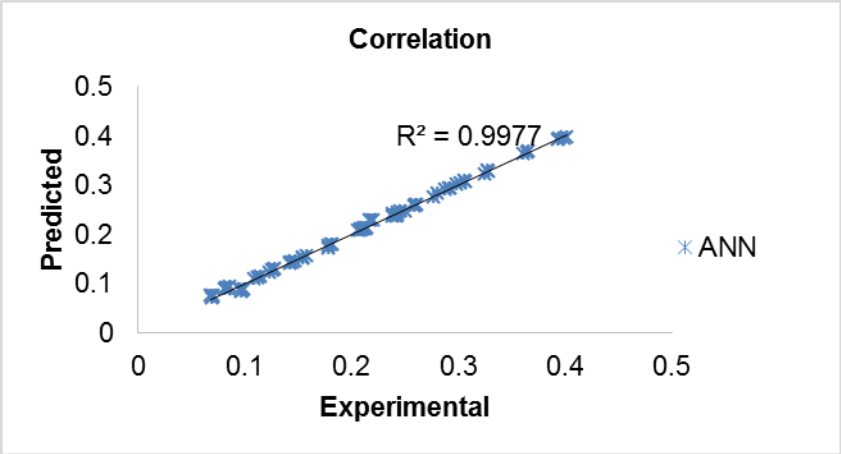


Figure 3.24: Correlation plot of ANN for 3- parameters

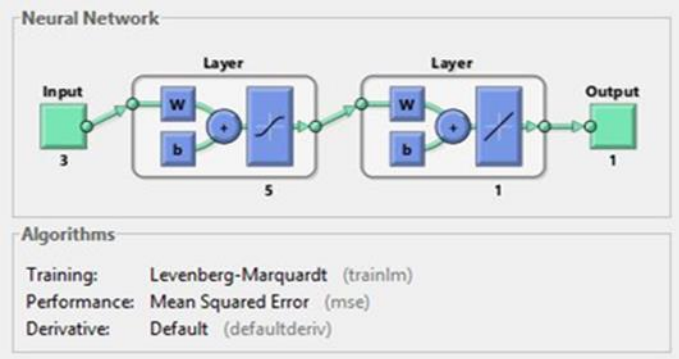


Figure 3.25: The architecture of ANN for 3-parameter

Similarly, the correlation plot for Bayesian regulation used in addition to back propagation was shown in Figure 3.26. The architecture of BR-ANN is shown in Figure 3.27

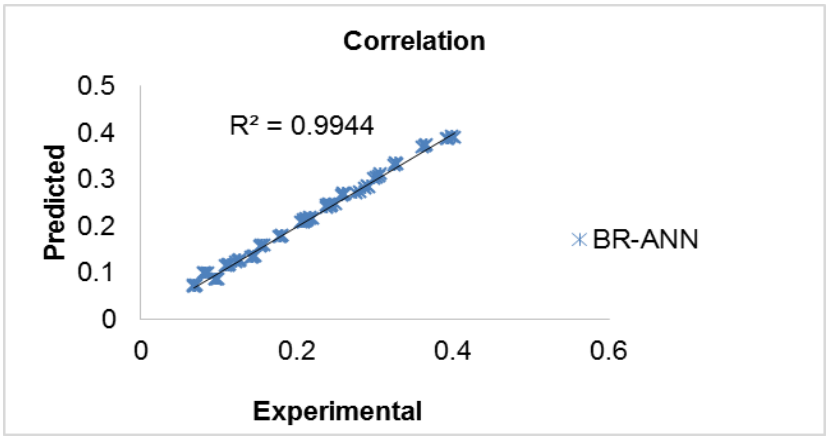


Figure 3.26: Correlation plot of BR-ANN for 3-parameters

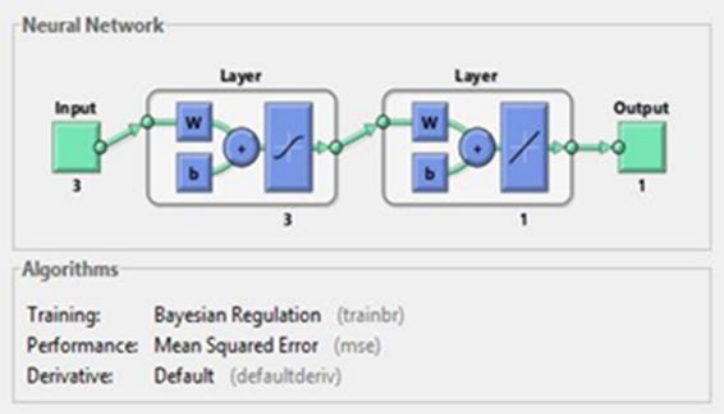


Figure 3.27: The architecture of BR-ANN for 3-parameter

The results showed that there was satisfactory goodness of fit for the model constructed. On applying the optimization technique of genetic algorithm, the parameters and the predicted yield was obtained for both the Neural Network Models as shown in Table 3.10

Table 3.10: Settings of parameters obtained from GA with the predicted and experimental yield and percent deviation from predicted value

Model	pH	Temp	Ip	I	S	Predicted yield	Experimental yield	% variation
Single layer ANN	5.00253	28.4171	49.0408	1.45082	1.98817	0.41421	0.451	8.8824938
Single layer ANN-with Bayesian Regularization	5.1402	25.0044	53.0863	1.45556	1.99665	0.41484	0.422	7259666

The final column represents the deviation in the yield from the experimental value. The % deviation in the value is less than 10% for all the models obtained. Further it can be noted that by using the Bayesian Regularized ANN, the % deviation is also reduced drastically which emphasizes the point that the BR-ANN is good in generalization capability.

Taguchi method was applied for the parameters. The percentage contribution of each parameter was calculated and shown in Table 3.11

Table 3.11: Percentage contribution of each parameter

Parameters	% significance
pH	3.88939
Temperature	23.87197
Incubation Period	32.58957
Inoculum volume	3.56124
Substrate volume	36.08783

According to these data, temperature, substrate volume and incubation period were more effective than the other factors, and pH of the medium and inoculum volume showed the least impact factor. Pooling of factors was carried out to understand whether leaving out the factors with little impact can change lipase production significantly or not. Hence pooling of the values of pH 5 and inoculum volume of 1.45 ml by keeping them constant without adjustment, the ANN and BR-ANN models were built with only 3 input parameters and one output.On applying the optimization technique of genetic algorithm, the parameters and the predicted yield was obtained for both the Neural Network Models. Table 3.12 represents the parameters and the predicted yield, experimental yield and the % deviation from the predicted value [181]. By classical

method of optimization the enzyme activity was found to be 37 U/ml whereas with statistical approach the activity was found to be 38.5 U/ml).

Table 3.12: Predicted and experimental yield and percent deviation from predicted value.

Model	Parameters			Yield from predicted data	Yield from experiment	% variation
	T	I.P	S			
Single layer ANN	29.0957	45.2212	2	0.40003	0.469	17.24062075
Single layer ANN-with Bayesian Regularization	28.8248	44.1116	1.99989	0.37752	0.471	24.76028014

Where T= temperature, I.P= incubation period, S= substrate volume

It was observed that the experimental yield had increased drastically, when compared to one variable at a time approach which suggested that the removal of insignificant parameters and training the network had improved the performance of the network. It was also observed that % deviation had also increased. This may be due to neglecting the effect of pH and inoculum in the modeling of neural network. It was further seen that the yield obtained by the setting of BR-ANN was better than the ANN model.

3.12 Conclusion

Lipases find a wide range of application in different industries like food, flavor, textile, degumming, cosmetic and biomedical. Thus, ahigher demand for the enzyme makes higher yield an essential need and the yield can be increased with more favorable cultural conditions. Many reports in the literature reveals that fungal lipases offer a distinct advantage over bacterial enzymes in terms of ease of downstream processing but there are several disadvantages like

- Time taken for its growth
- Chances of contamination more
- Difficult in bringing selective mutations due to large genome when required for strain improvement.

Due to above reasons probiotic lactic acid bacteria were considered for preliminary work which are generally regarded as safe and less reported in literature.

Optimization plays a very important role in fermentation processes for increased production of the enzyme.The lipase activity with classical method of optimization was 32 U/ml from co-culture of *L. lactis* and *L. brevis*, 35 U/ml for *L. lactis* and *L. plantarum* and37 U/ml for *L. brevis* and *L. plantarum* whereas with the above statistical approach the activity

was found to be 33.5 U/ml from co-culture of *L. lactis* + *L. brevis* and 36 U/ml from *L. lactis* + *L. plantarum* and 38.5 U/ml for *L. brevis* and *L. plantarum* which showed more than 2 fold increase in the lipase production.From the above results it was evident that statistical approach was more beneficial in the production of lipase from both single and co-cultures of different lactic acid bacteria when compared to the classical OFAT (one factor at a time) optimization. To the best of our knowledge, optimization of process parameters for lipase production from single culture of *Lactococcus lactis* was the first report in the literature though reports are on esterase genes. Similarly it was the first report on production of lipase from co-culture of lactic acid bacteria.

These optimization results paved way for further strain improvement studies for an increased lipase production, which has been reported in the subsequent chapters.

Strain improvement by mutagenesis

4.1 Introduction

In the previous chapter it was seen that the product yield in a fermentation process could be increased by manipulation of the parameters relating to fermentation. Besides these, there are other techniques to increase productivity. Strain can be improved either by mutation, by protoplast fusion or by using the recombinant DNA technology. Notable among these are genetic methods i.e. mutation [182]. The use of mutation is a valuable tool for strain improvement of many enzyme producing organisms. Mutations are the raw material of evolution, providing the changes on which selection can act. Mutagenesis is the process of formation of a mutant organism [183]. The term mutant refers to an organism in which either the base sequence of DNA or the phenotype has been changed. Mutation leads to discontinuous hereditary changes of genetic material which can take place both spontaneously and by induction of mutagenic agents [184]. There are many different ways that DNA can be changed, resulting in different types of mutation like substitution, insertion, deletion and frameshift [185-186]. A natural or human-made agent (physical or chemical) which can alter the structure or sequence of DNA [187] is a mutagen.

The rate of mutation can be increased by mutagens. The notable mutagenic agents are ionizing radiations (e.g. x-rays, gamma rays, non-ionizing agents (e.g. Ultraviolet radiation) and chemical agents (namely Ethidium bromide (EtBr), Nitrosoguanidine (NTG), Diethyl sulphate (DES), Nitrous acid (HNO_2), Mustard gas). Ionization radiation induces single or double strand breakage leading to deletion/ structural changes in DNA [188]. Ultraviolet radiation which, when absorbed by the DNA causes a cross link to form between certain adjacent bases inducing pyrimidine dimmers [189]. It causes transversions, deletions

and frame shift transitions. UV radiation is less energetic, and therefore non-ionizing, but its wavelengths are preferentially absorbed by bases of DNA and by aromatic amino acids of proteins, so it, has important biological and genetic effects. Chemical mutagens change the sequence of bases in a DNA gene in a number of ways by duplication, deletion and additions [190]. The choice and dosage of mutagen plays an important role in strain improvement by mutating certain parts of the genome.

The induction of mutation by UV in *Pleurotus pulmonarius* with enhanced laccase production has been reported and highest activity of 2.5 U/ml was obtained than compared to wildstrain [191]. A *Pseudomonas* mutant with enhanced lipase production was also obtained by UV and nitrosoguanidine (NTG) treatment [192]. Another group of workers [193] obtained an UV induced mutant which is capable of maltose utilization from a sucrose utilizing strain of *Saccharomyces cerevisiae*. Mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine for cellulase production from *Bacillus* sp. (MTCC10046) produced large amounts of the enzyme than the wild type [194]. Mutants of *Rhizopus* sp. were also isolated with enhanced cellulase production using UV rays as mutagenic agent [195]. Another group of scientists [196] isolated and characterized mutant of *Schwanniomyces gastellii* for amylase production.

The strain *Trichoderma reesei* Rut C-30 showed enhanced cellulase production after successive treatment with NTG for 6 hrs followed by UV [197] for 15minutes. Lipase from *Aspergillus japonicas* MTCC 1975 showed 127%, 177%, 276% higher lipase yield than parent strainusing UV, HNO_2 and NTG mutagens [198]. Ultra-violet (UV) irradiation, ethyl methane sulfonate (EMS) and ethidium bromide were used as mutagens for production of industrially important fibrinolytic protease from *Bacillus cereus* GD 55 strain [199].

The process of mutation involves two steps, namely, the treatment of wild organism with mutagen and then the isolation of mutant prior to testing and selection. In addition to

random testing of survival colonies, the most commonly used criterion for selection is morphological change. Other rational selection methods are analogue-resistant-mutants [200-201], auxotroph, biochemical mutants etc. In the present chapter, the detailed procedure for isolation of mutant has been discussed and their characteristics have been compared with the parent strain.

4.2 Mutagenesis by ultraviolet (UV) irradiation

For mutation by UV radiation, all the bacterial single cultures like *Lactococcus lactis, Lactobacillus brevis, Lactobacillus plantarum* and co-cultures like *Lactococcus lactis* + *Lactobacillus brevis, Lactococcus lactis* + *Lactobacillus plantarum* and *Lactobacillus brevis* + *Lactobacillus plantarum* of 1 ml each were taken in tubes and one control for each was kept aside and the rest of the tubes were exposed to UV at an intensity of 254 nm irradiation [145,202] for about 30, 60, 90 and 120 min. Immediately after exposure to UV, 20 microlitres from 1ml of the sample was spread on the tributyrin agar plate and incubated at their respective optimum temperatures obtained in chapter 3. After incubation, the enzyme activity was done using *p*-nitrophenyl palmitate as substrate at 410 nm. Maximum enzyme activity was obtained with 120 min of UV exposure as shown in Figure 4.1

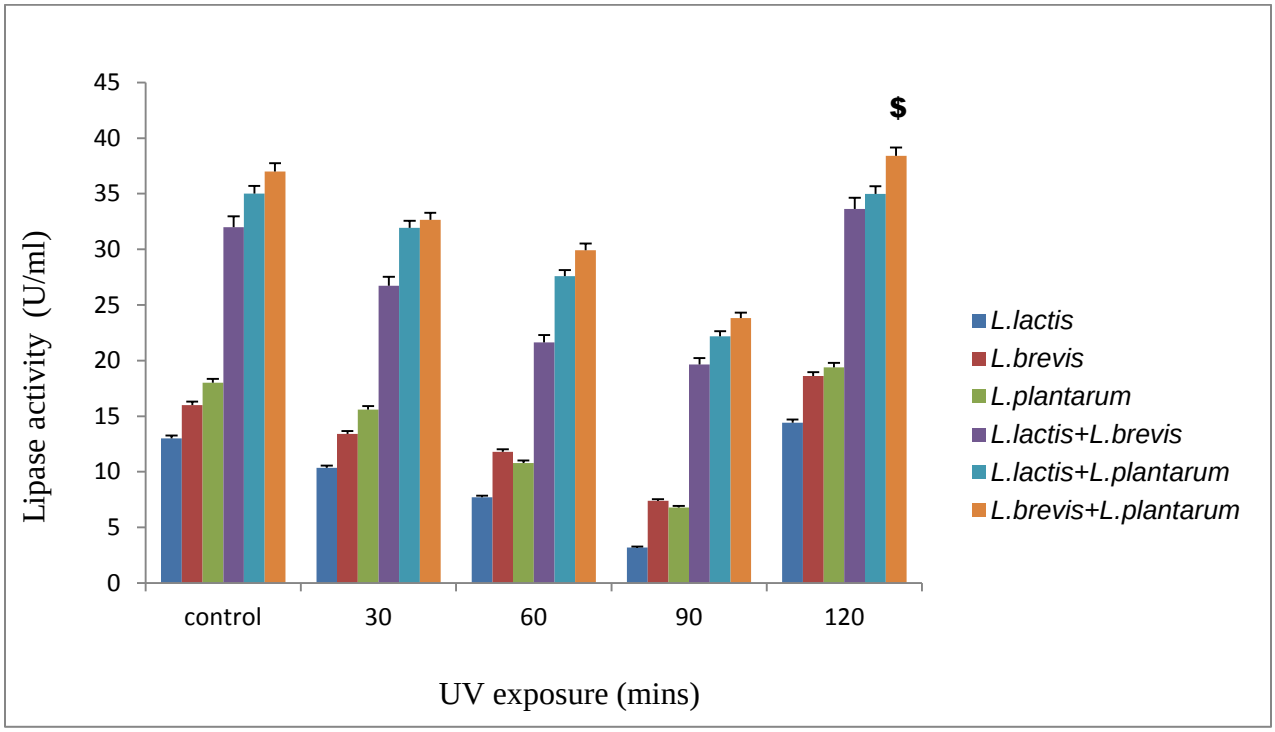


Figure 4.1: Effect of UV

Data was mean standard deviation of 9 replicates from 3 experiments ($p \leq 0.05$). $^{\$}$ represents maximum enzyme activity obtained at 120 min of UV.

4.3 Mutagenesis by heat treatment

In this experiment of mutagenesis by heat treatment, single cultures like *L. lactis*, *L. brevis*, *L. plantarum* and co-cultures like *L. lactis* + *L. brevis*, *L. lactis* + *L. plantarum* and *L. brevis* + *L. plantarum* of 1 ml each were taken in a sterilized test tube and was heat-treated to 60 ^{o}C for varying time periods from 35–65 minute at an interval of 5 min. This process was done for all the combinations plates (for 35, 40, 45, 50, 55, 60 and 65 min). All the plates were kept in dark for half an hour and were then incubated at 37 ^{o}C. The colonies formed are grown in 50 ml of lipase assay medium containing peptone (0.5%), yeast extract (0.3%), NaCl (0.25%), $MgSO_4$ (0.05%) and olive oil (substrate)and incubated. After incubation, the enzyme activity was done using *p*-nitrophenyl palmitate as substrate at 410 nm.

With the increase in the time period of heat treatment, there is decrease in the lipase activity as shown in Figure 4.2.

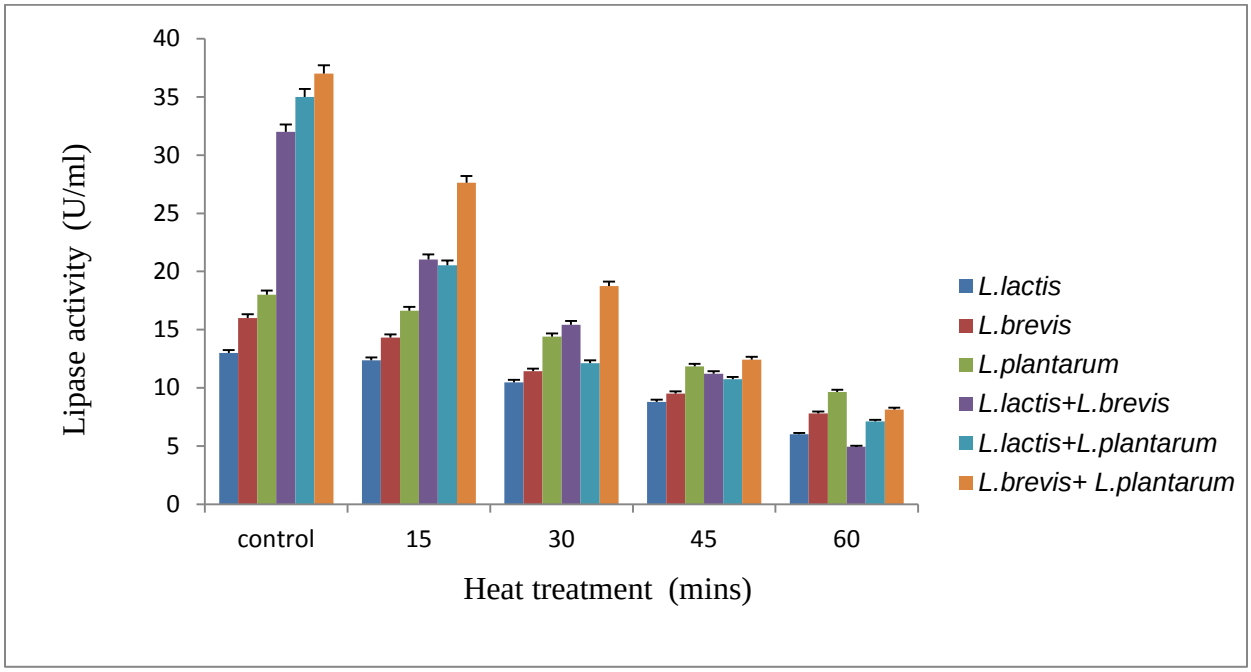


Figure 4.2: Effect of heat treatment

Data was mean standard deviation of 9 replicates from 3 experiments ($p \leq 0.05$).

4.4 Mutagenesis by EtBr

The chemical mutagen, EtBr acts by intercalating between the base pairs of a DNA molecule leading to the change in its flexibility. In this experiment for checking mutagenesis, single cultures like *L. lactis, L. brevis, L. plantarum* and co-cultures like *L. lactis* + *L. brevis, L. lactis* + *L. plantarum* and *L. brevis* + *L. plantarum* were treated with the chemical mutagen EtBr in different concentrations of 5, 10, 15 and 20 µlts and then placed on tributyrin agar. The colonies formed are grown in 50 ml of lipase assay medium containing peptone (0.5%), yeast extract (0.3%), NaCl (0.25%), $MgSo_4$ (0.05%) and olive oil (substrate)and incubated. After incubation, the enzyme activity was measured using P-nitrophenyl palmitate as substrate at 410 nm.

The enzyme activity decreased with the increase in the concentration of EtBr as shown in Figure 4.3.

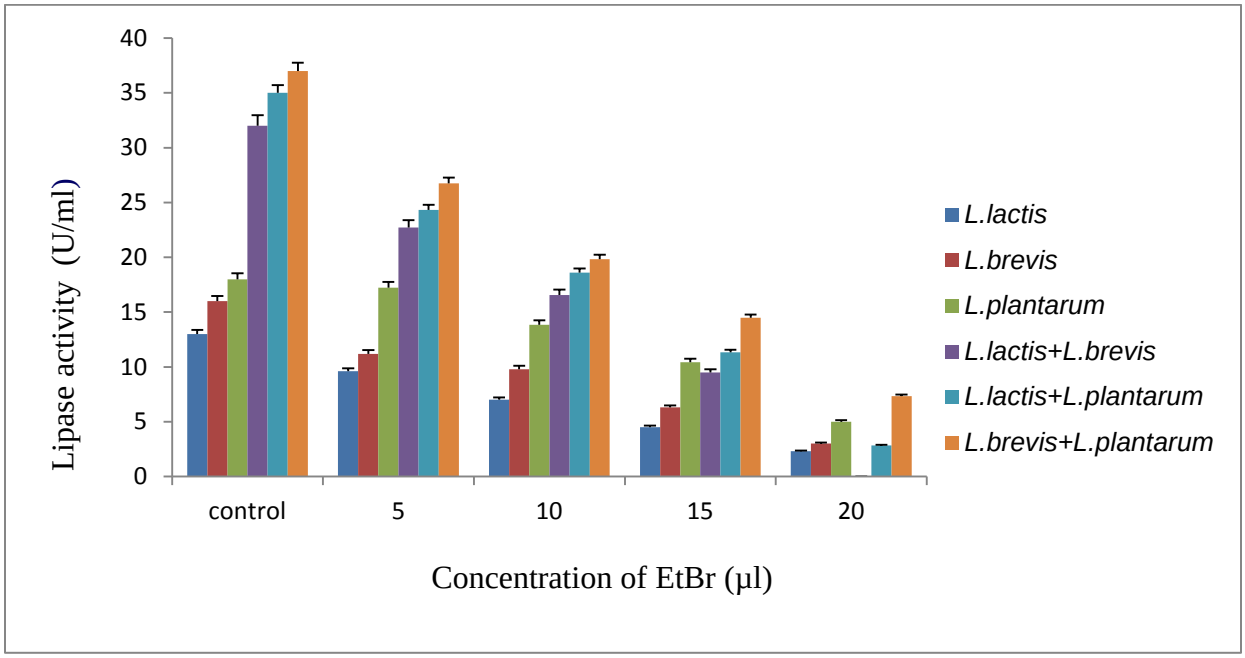


Figure 4.3: Effect of Ethidium Bromide

Data was mean standard deviation of 9 replicates from 3 experiments ($p \leq 0.05$).

4.5 Dry cell weight determination

The mutant bacterial culture samples containing the single cultures like *L. lactis*, *L. brevis*, *L. plantarum* and co-cultures like *L. lactis* +*L. brevis*, *L. lactis* + *L. plantarum* and *L. brevis* + *L. plantarum* were centrifuged at 13,000 rpm for 15 min at 4 ^{o}C. The pellet was suspended in distilled water and centrifuged. Then the cells were dried and weighed. The biomass was expressed as mg of cell dry weight per milliliter [203] (mg cell dry weight/ml). The dry weight obtained by mutant single and co-cultures are shown in Table 4.1.

Table 4.1: Dry weight of mutant single and co-cultures

Time (in hrs)	*L. Lactis*		*L. brevis*		*L. plantaram*		*Lc + LBb*		*Lc + LBp*		*LBb+ LBp*	
	Dry weight (mg)	Enzyme activity (U/ml)	Dry weight (mg)	Enzyme activity (U/ml)	Dry weight (mg)	Enzyme activity (U/ml)	Dry weight (mg)	Enzyme activity (U/ml)	Dry weight (mg)	Enzyme activity (U/ml)	Dry weight (mg)	Enzyme activity (U/ml)
12	0.165± 0.001	8.9± 0.10	0.21± 0.01	13± 0.11	0.23± 0.01	13.9± 0.12	0.42± 0.001	16.9± 0.125	0.48± 0.002	21± 0.136	0.92± 0.001	28± 0.17
24	0.228± 0.001	10.6± 0.123	0.26± 0.005	14± 0.12	0.31± 0.01	15± 0.13	0.46± 0.01	20± 0.13	0.5± 0.002	22.4± 0.142	0.96± 0.002	29.64± 0.19
36	0.251± 0.001	14.2± 0.113	0.29± 0.015	15.9± 0.17± 0.18	0.35± 0.001	17.8± 0.14	0.49± 0.02	23.5± 0.14	0.57± 0.001	31.4± 0.156	0.100± 0.001	35.45± 0.21
48	0.187± 0.001	13.5± 0.112	0.34± 0.001	16.7± 0.15	0.36± 0.02	19.2± 0.143	0.60± 0.015	25.8± 0.142	0.69± 0.001	*35± 0.210	1.109± 0.002	*39± 0.27
60	0.118± 0.001	12.4± 0.110	0.29± 0.001	15± 0.171	0.39± 0.01	17.3± 0.136	0.67± 0.025	28.5± 0.185	0.77± 0.002	33.8± 0.204	0.99± 0.003	36.91± 0.265
72	0.087± 0.001	12.1± 0.102	0.27± 0.01	10.9± 0.123	0.29± 0.002	16.8± 0.128	0.78± 0.015	*34± 0.2	0.88± 0.025	29.8± 0.198	0.95± 0.002	34.23± 0.253
84	0.058± 0.001	11± 0.1	0.25± 0.015	10± 0.110	0.27± 0.001	14.46± 0.121	0.70± 0.015	21.7± 0.192	0.79± 0.010	25.8± 0.19	0.89± 0.001	30.56± 0.241
96	0.036± 0.001	10.9± 0.1	0.21± 0.010	9.8± 0.10	0.25± 0.001	11.73± 0.11	0.63± 0.015	18.5± 0.187	0.70± 0.020	21.63± 0.17	0.82± 0.001	28.56± 0.232

Experiments were conducted in triplicate and results were the average of these three independent trials.* represents significant value ($p \leq 0.05$).

4.6 Comparative study of parent and mutant strains

4.6.1. Biochemical tests

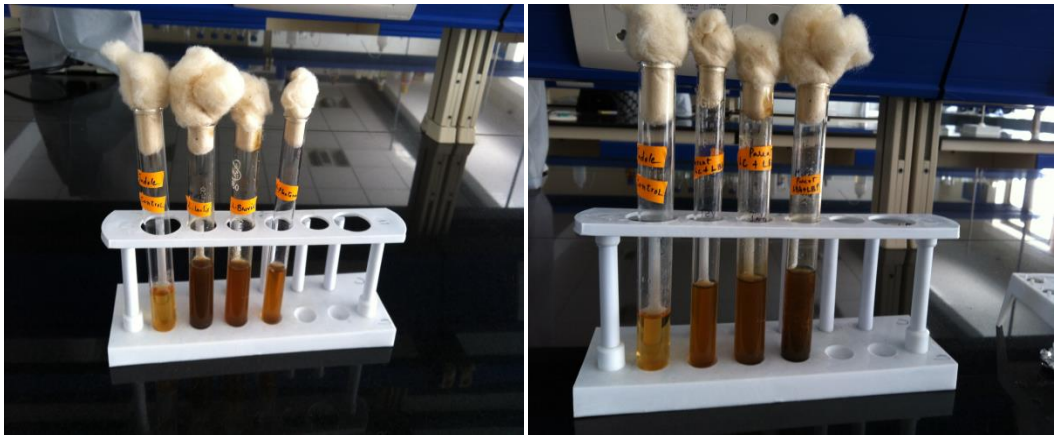
The mutant isolates were characterized by biochemical tests using Bergy's manual of determinative Bacteriology [204]. Both the parental and mutant isolates were tested with gram staining, followed by IMViC tests, followed by amylase, oxidase and catalase test to characterize the strains in terms of their phenotypic and physiological characters.

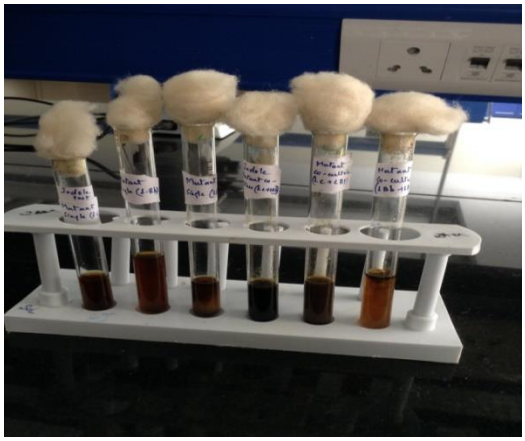
4.6.1.1 Indole test

The ability of the organism to convert tryptophan into indole in presence of tryptophanase enzyme is the main principle behind this biochemical test. All the bacterial single and co-cultures were grown in sterile tryptone broth. After 24 hrs incubation, 2-3 drops of kovacs reagent was added. Para di amino benzaldehyde present in kovacs reagent reacts with indole to form a rosindole dye imparting red colour. The organisms which convert tryptophan present in the media into indole are indole positive and which do not is indole negative.

All single and co-cultures (both parent and mutants) were found to be indole negative as shown in Figure 4.4

(a) **(b)**





(c)

Figure 4.4: Indole test (a) parent single cultures (b) parent co-culture (c) mutant single and co-cultures

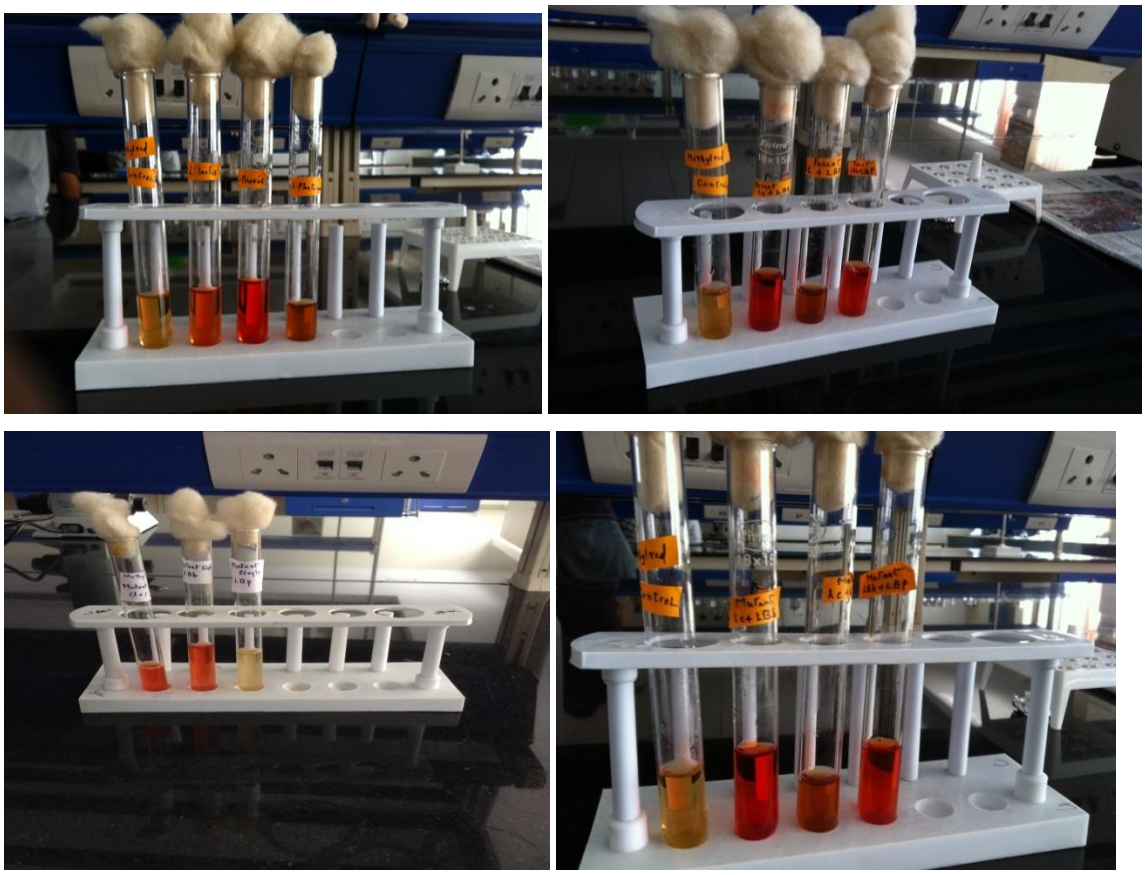
4.6.1.2 Methyl red and Vogesproskeur (MR-VP) test

Some organisms during fermentation of glucose produce large amounts of acids and other organisms produce neutral end products (acetyl methyl carbinol). To differentiate this MR-VP test is done on MR-VP broth. If the organism produces substantial amount of acids, on addition of methyl red, a pH indicator the medium will remain red indicating the acidic pH. If the medium turns yellow then it shows negative test due to increased pH to 6 or above. Similarly for Vogesproskeur test, barrits reagent is added into medium.

The single parent culture *L. brevis* and mutant single culture *L. plantarum* are positive to methyl red test. Parent co-cultures *L. lactis +L. brevis* and *L. lactis + L plantarum* were methyl red positive as shown in Figure 4.5.

Similarly except parent single culture *L. plantarum* and mutant single culture *L. lactis* all the other parent and mutant single and co-cultures were positive to vogesproskeur test as shown in Figure 4.6.

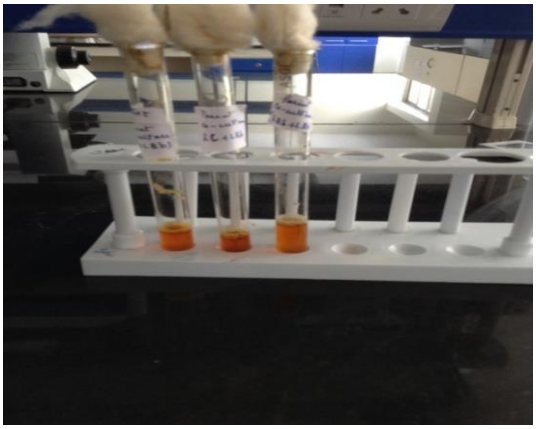
(a) **(b)**

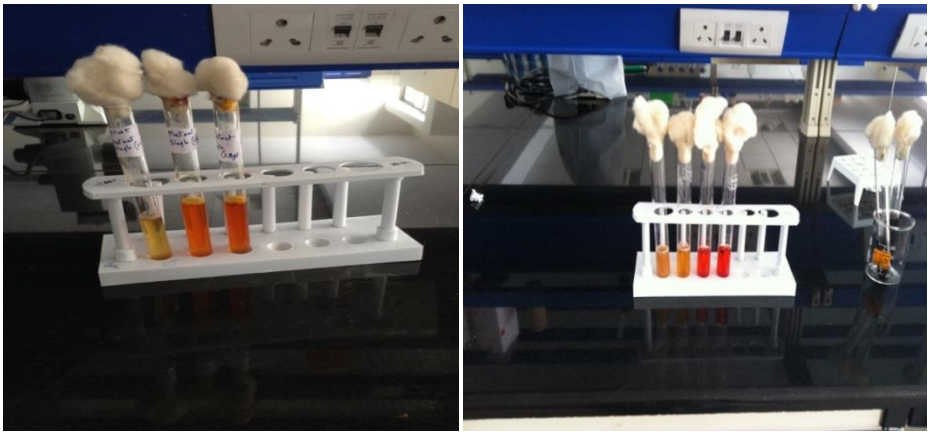


(c) **(d)**

Figure 4.5: Methyl red tests (a) parent single culture (b) parent co-culture (c) mutant single culture (d) mutant co-culture

(a)





(b) **(c)**

Figure 4.6: Vogesproskeur test (a) parent co-culture (b) mutant single cultures (c) mutant co-cultures

4.6.1.3 Citrate test

Certain organisms have ability to utilize citrate as sole source of carbon. In the above test all the parent and mutant single and co-cultures were grown on agar plates containing simmon's citrate agar. If the organisms have capacity to utilize citrate as carbon source, the medium changes into blue colour as the pH becomes alkaline following citrate utilization. There no change in pH and color of the medium when the citrate is unutilized in the medium.

Except the parent single culture of *L. lactis* and mutant co-culture *L. lactis* and *L. brevis* all were citrate positive as shown in Figure 4.7.

(a) **(b)**

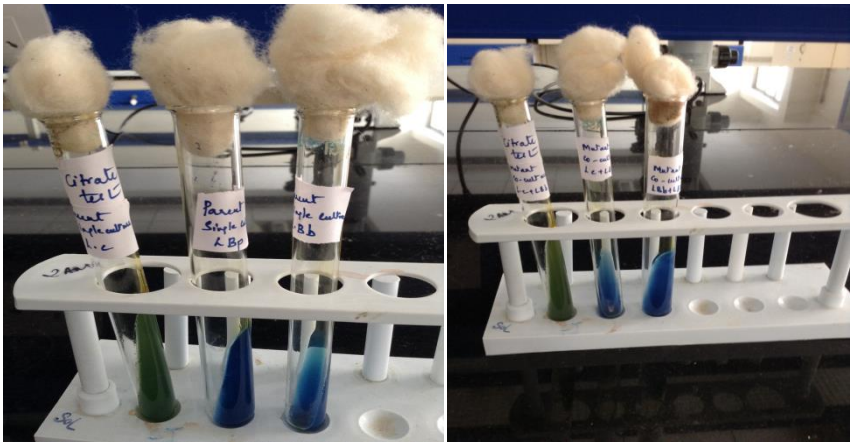


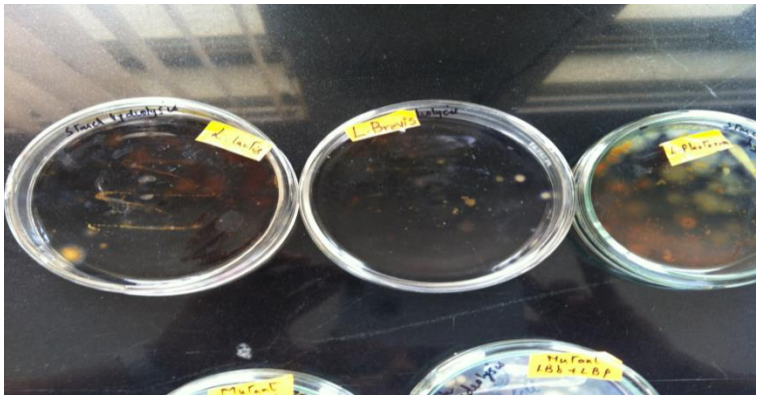
Figure 4.7: Citrate test (a) parent single culture (b) mutant co-culture

4.6.1.4 Starch hydrolysis

The organisms which have capacity to utilize starch, a polymer of sugar as sole source of carbon have an ability to degrade when grown in starch agar medium. The organisms are inoculated into the plates and incubated for 24 hrs and after incubation iodine solution is added into the plate. Blue-black colour appears due to formation of starch-iodine complex. If no colour appears streaked culture area become clear it indicates the degradation of starch occurs due to production of amylase.

The mutant single culture *L. lactis* and mutant co-cultures, *L. lactis* + *L. brevis and L. brevis* + *L. plantarum* were positive to the test as shown in Figure 4.8.

(a)



(b)

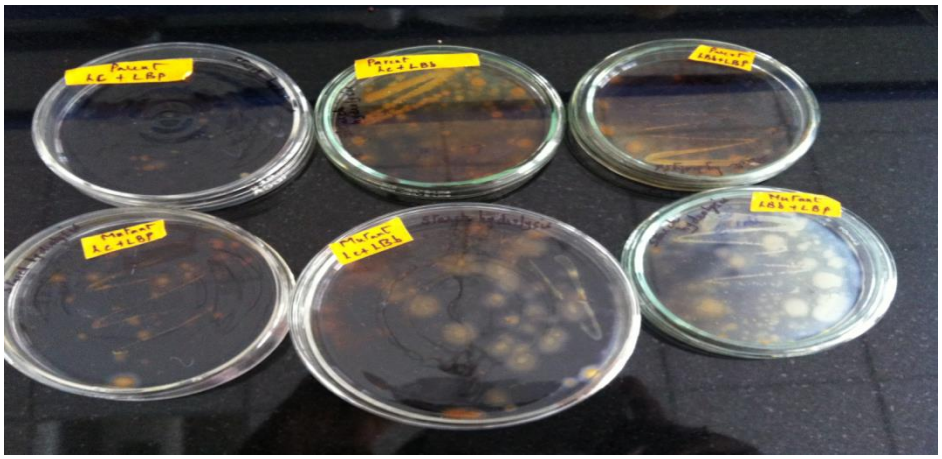


Figure 4.8: Starch hydrolysis (a) parent single cultures (b) parent and mutant co-cultures

4.6.1.5 Oxidase test

All the parent and mutant single and co-culture were grown on tripticase soy agar and after incubation, p-aminodimethylanilinoxalate was added. If it donates electron to cytochrome C, then it gets oxidized and first becomes pink, later changes to red and black showing that they were oxidase positive. All the parent and mutant single and co-culture were oxidase negative as shown in Table 4.2.

4.6.1.6 Protease test

All the parent and mutant single and co-cultures were grown on nutrient gelatin agar/ casein agar. If the organisms hydrolysed casein/gelatin in presence of protease enzyme then they were positive forming clear zone of colonies or else they were negative. Except parent single culture *L. lactis* and mutant single culture *L. brevis,* all the cultures were protease positive as shown in Table 4.2.

4.6.1.7 Nitrate reduction test

Certain bacteria use nitrogen in place of oxygen as an external terminal electron acceptor. All the cultures were grown on nitrate broth after incubation; 3 drops of solution A (suphanilic acid) and solution B (dimethyl-alpha-napthyalamine) were added. Appearance of red colour indicated nitrate reduction. But if there is no red colourformation then the test is found to be negative. This can further be confirmed by adding zinc dust to the tube where if red colour appears then the test is negative. Single parent culture *L. brevis* and parent and mutant co-cultures, *L. lactis* + *L. brevis* and *L. lactis* + *L. plantarum* were positive to the test as shown in Table 4.2.

4.6.1.8 Catalase test

Some aerobes and facultative anaerobes show catalase activity. They utilize oxygen and produce hydrogen peroxide which is toxic to their cell. These organisms produce an

enzyme called catalase which converts hydrogen peroxide into water and oxygen. A colony of parent and mutant single cultures and co-cultures were taken onto slides and hydrogen peroxide was added. If bubbles appeared then the organisms were said to be catalase producers. Except mutant co-culture of *L. lactis* + *L. plantarum* all the cultures were catalase negative as shown in Table 4.2. The response to different biochemical tests by parent and mutant cultures were shown in the Table 4.2. All the experiments were done in triplicates.

Table 4.2: Biochemical activity profile of parent and mutant strains

Biochemical tests	Parent single culture.			Mutant single culture			Parent co-culture			Mutant co-culture		
	Lc	*LBb*	*LBp*	*Lc*	*LBb*	*LBp*	*Lc+ LBb*	*Lc+ LBp*	*LBb+ LBp*	*Lc+ LBb*	*Lc+ LBp*	*LBb+ LBp*
Indole	-	-	-	-	-	-	-	-	-	-	-	-
Methyl red	-	+	-	-	-	+	+	-	+	+	-	+
Vogesproskeur	+	+	-	-	+	+	+	+	+	+	+	+
Citrate	-	+	+	+	+	+	+	+	+	-	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-
Protease	-	+	+	+	-	+	+	+	+	+	+	+
Nitrate reduction	-	+	-	-	-	-	+	-	+	+	-	+
Starch hydrolysis	-	-	-	+	-	-	-	-	-	+	-	+
Catalase	-	-	-	-	-	-	-	-	-	-	+	-

Where *Lc= Lactococcus lactis, LBb= Lactobacillus brevis, LBp= Lactobacillus plantarum, Lc+LBb = Lactococcus lactis* and *Lactobacillus brevis*, *Lc+LBp= Lactococcus lactis* and *Lactobacillus plantarum*, *LBb+LBp= Lactobacillus brevis* and *Lactobacillus plantarum*

Where + = positive, - = negative.

4.6.2. Response to Antibiotics

The response to various antibiotics by the mutant was compared with that of the parent strain. This was done by observing their growths in Muller Hilton agar plates in presence of various concentrations of different antibiotics. The results of this study have been presented in the Table 4.3. All the experiments were done in triplicates. Antibiotics acts by inhibiting the cell wall synthesis, protein synthesis, nucleic acid synthesis and metabolic processes.

4.6.2.1 Mode of action of Ampicillin

It is β-lactum antibiotic which is irreversible inhibitor of enzyme transpeptidase which is important for bacteria for cell wall synthesis leading to cell lysis.

4.6.2.2 Mode of action of Amoxicillin

It inhibits the cross linking of peptidoglycan polymer chains of cell wall of gram positive and gram negative bacteria.

4.6.2.3 Mode of action of Penicillin

It is β-lactum antibiotic which acts by inhibiting the formation of peptidoglycan cross-links in the bacterial cell wall.

4.6.2.4 Mode of action of Tetracycline

It inhibits protein synthesis by blocking the attachment of charged aminoacyl-tRNA to the A site on the ribosome.

4.6.2.5 Mode of action of Streptomycin

Streptomycin binds to the small 16S rRNA of the 30S subunit of the bacterial ribosome, interfering with the binding of formyl-methionyl-tRNA to the 30S subunit leading to codon misreading, inhibition of protein synthesis and death of microbial cells.

Table 4.3: Response to various antibiotics by parent and mutant strains.

Antibiotics	Concentration (µg/ml)	Parent single culture			Mutant single culture			Parent co-culture			Mutant co-culture		
		Lc	*LBb*	*LBp*	*Lc*	*LBb*	*LBp*	*Lc+LBb*	*Lc+LBp*	*LBb+LBp*	*Lc+LBb*	*Lc+LBp*	*LBb+LBp*
Ampicillin	100	++	++	-	+	+	++	++	+	++	++	+	++
	200	++	++	-	++	+	+	++	+	+	++	+++	+++
	400	++	++	-	+	+	+	++	+	+	++	+	+
Amoxycillin	100	++	++	++	+	+	+	++	++	++	++	+	++
	200	++	++	++	+	+	+	++	++	++	++	+	++
	400	++	++	++	++	++	++	++	++	++	++	+	++
Penicillin	100	+	+	+	-	-	-	+	+	+	-	-	-
	200	+	+	+	-	-	-	+	+	+	-	-	-
	400	++	++	-	-	-	-	++	+	+	-	-	-
Tetracycline	100	++	+++	-	+	+	+	++	++	++	+	++	++
	200	–	+	-	-	+	+	+	+	+	–	+	+
	400	–	+	-	-	+	+	+	+	+	–	+	+
Streptomycin	100	-	+	+	+	++	+	+	++	++	++	+++	++
	200	-	+	+	+	+	+	+	+	++	++	++	++
	400	-	+	+	+	+	+	+	+	+	+++	+++	+++

Where *Lc= Lactococcus lactis, LBb= Lactobacillus brevis, LBp= Lactobacillus plantarum.*

Where +++ = more resistant, ++ = intermediate, += resistant, - =sensitive

4.7 Discussion

The mutation and screening of industrially useful microorganisms are important for the successful development of the various strains required in the fermentation industry.

With the increase in the time period of exposure to the UV radiation from 30-120 min, there is decrease in enzyme activity. But for 120 min incubation, there is increase in the lipase activity of the three mutant co-culture combinations showing *L. lactis + L. brevis as* 34 U/ml, *L. lactis + L. plantarum as* 35 U/ml and *L. brevis + L. plantarum as* 39 U/ml respectively when compared to the individual normal controls whose activities were 32 U/ml for *L. lactis + L. brevis*, 35 U/ml for *L. lactis + L. plantarum* and 37 U/ml for *L. brevis + L. plantarum* [158]. One reason could be reversions or reverse mutations that change the new phenotype back to the original or due to multiple mutations. Only 2 U/ml increase in enzyme activity was observed when compared to parent strains.

With the increase in the time period of heat treatment from 5-60 minutes at 60 ^{o}C, there is decrease in the lipase activity. The reason could be denaturation of the protein due to increase in temperature. Similarly with increase in concentration of ethidium bromide from 5-20 µl, there is decrease in enzyme activity which might be due to intercalation of the mutagen with DNA preventing protein synthesis.

From Table 4.1 it is evident through cell dry weight determination that among single cultures *L. plantarum* and co-cultures *L. brevis + L. plantarum* showed highest activity.

Analysis on gelatin agar plate confirmed the better ability of the mutant to produce protease in addition to lipase enzyme. In case of starch agar plate, after iodine reaction, the pale yellow zone was maximum for the mutant showing the amylase hyper production efficiency of the mutant co-cultures (*L. lactis + L .brevis)* and *(L. brevis + L. plantarum*) as shown in Table 4.2. The mutants were found to be a hyper producer of protease and amylase

too, apart from lipase. From this it can be inferred that the increased rate of growth during initial stages of their lifecycle may be due to reduction or complete absence of their lag phases.

The importance of antibiotic sensitivity test is to see sensitivity or susceptibility of bacteria to different antibiotics. The mutant single and co-cultures were found to be sensitive towards penicillin and resistant towards ampicillin and streptomycin as shown in Table 4.3.

4.8 Conclusion

UV irradiated mutant showed an increase in lipase activity of 2 U/ml over heat and ethidium bromide treatments compared to the parent strain. Though it has no special effects on the morphology of the strains but there are some effects on the biochemical characteristics of the organism. This suggested that there could have taken place some change in the composition i.e. in amino acid sequence of the parent organism resulting in the mutant strain. The improved strains obtained after mutation were also a hyper producer of protease in addition to lipase. Therefore, utilization and exploitation of this mutant would be sensible for large-scale production of industrial enzymes.

Although mutant strains possess novel properties, there was not much improvement in lipase activity compared to parent strain; hence an alternative approach was used to improve the strain by recombinant DNA technology which is reported in the next chapter.

Heterologous expression of lipase and its purification study

This chapter includes heterologous expression of lipase gene in *E. coli* BL21 *(DE3) pLysS* and purification of recombinant protein using amylose column and native protein obtained after optimization by ammonium sulphate precipitation followed by sepharose column chromatography.

5.1 Introduction

Microbes serve as an excellent source of lipases compared to plant and animal lipases because of their rapid growth, limited space for cultivation, withstanding various temperatures and easy genetic manipulation. They have capability to generate high yields desirable for various applications [205].

Higher rates of protein production, product quality and host cell density are important aspects in production of industrial products. As cultivation of some microbes under laboratory conditions may not result in increased yield of lipase, techno economic feasibility of lipase production is a major challenge across the globe. In this regard, use of heterologous expression system aids in enhancing the expression level of lipase genes in various hosts than that in their corresponding native host [206-209]. In heterologous gene expression, foreign gene was expressed by the host organism for increased production of desired products.

An alkaline lipase gene from *Penicillium cyclopium* PG37 was cloned and expressed in *Pichia pastoris.* After expression there was increase in lipase activity up to 407 U/ml. The recombinant alkaline lipase showed the highest activity at pH 10.5 and 25 ^{o}C [210]. Two genes encoding for cold-active lipolytic enzymes, Lip-1452 and Lip-948 were isolated from *Psychrobacter* sp an Antarctic bacteria. The genes were screened, cloned into vector pCold III and expressed in *E. coli* BL21 (DE3) cells [211]. 3.7 U/ml lipase activity was observed

with pCold III + Lip-948 and low activity with pCold III+Lip-1452. Lipase Lip2 gene of *Pleurotus sapidus*, a basidiomycete was expressed in *Escherichia coli*. The heterologous expression led to the high level production of recombinant protein, mainly as inclusion bodies [212]. Lipase gene from *Candida antarctica* ZJB09193 was cloned, and expressed in *Pichia pastoris* with the vector pPICZαA and a yield of 3g/l was obtained. The expressed enzyme was immobilized for biosynthesis of vitamin A esters [213]. A lipase gene from the fungus *Rhizomucor miehei* was expressed in methylotrophic yeast *Pichia pastoris* strain GS115 which showed the capability of converting micro algae oil to biofuel [214]. Another group of scientists isolated, cloned and expressed a lysophospho lipase gene from *Antrodia cinnamomea* in *E. coli* which was the first lysophospho lipase gene isolated and identified at molecular level [215].

In enzyme productiondownstream processing plays a very important role in the success of commercial biotechnological processes [216]. Purification of crude microbial product which contains mixture of various biochemical elements is one of the vital steps in downstream process. In most of the applications, the product must be obtained in a reasonably pure form. Therefore, the fermentation extract must be subjected to purification processes to eliminate unwanted components as far as possible and retain only the desired ones.

Many types of purification processes are available. They are the conventional techniques like gel-filtration, ion exchange chromatography, affinity chromatography, high performance liquid chromatography, ultrafiltration and electrophoresis [217-218]. All these techniques result in high levels of purity. Hence, these techniques are more suitable for pharmaceutical or research studies.

Lipase enzyme producing *Bacillus pumilus* RK 31 strain was isolated from oil contaminated soils. The enzyme was purified using ammonium sulphate, gel filtration

chromatography and ion exchange chromatography and a specific activity of 3525.6 U/mg was obtained with the three step purification process. The enzyme was also found to be tolerant to various organic solvents [219]. An alkaline tolerant lipase enzyme was produced from *Antrodia cinnamomea* BCRC 35396. The enzyme was purified by ammonium sulphate precipitation and DEAE-Sepharose chromatography. It showed a specific activity of 187.5 U/mg [220].

Lipase enzyme was produced and purified by ammonium sulphate precipitation and ion exchange chromatography from a bioluminescent organism *Vibrio fischeri* which showed a specific activity of 121 U/mg [221]. Different species of *Bacillus* (B1-B5) were isolated from oil spilled contaminated soils of Salem for lipase production. The enzyme was purified using ammonium sulphate precipitation followed by dialysis and column chromatography [222]. *Bacillus cereus* MSU AS, a lipase producing bacterium was isolated from the gut of a marine fish *Sardinella longiceps.* The enzyme was purified by ammonium sulphate precipitation followed by column chromatography and the lipase activity was found to be 48.82 U/ml with DEAE-cellulose column and 26.24 U/ml with Sephadex G-75 chromatography [223]. A thermostable lipase was isolated from *Staphylococcus xylosus*strain (wt-SXL2) and purified by Ni-NTA columns. The specific activity was found to be 6300 U/mg [224].

As discussed in the previous chapter the enzyme activity of single cultures after optimization was found to be 13 U/ml for *L. lactis,* 16 U/ml for *L. brevis* and 18 U/ml for *L. plantarum*. Similarly for co-cultures, the enzyme activity was 32 U/ml for *L. lactis* and *L. brevis,* 35 U/ml for *L. lactis* and *L. plantarum* and 37 U/ml for *L. brevis* and *L. plantarum.* The bacterial combination with highest activity was selected for heterologous expression and its application.

The present work deals with the heterologous expression of lipase gene from *L. brevis* and *L. plantarum* that produced highest enzyme activity and purification of the crude enzyme by amylose column.

5.2 Cloning and sequencing of lipase gene

5.2.1 Source of lipase gene and primer designing:

Lipase gene from probiotic Lactic acid bacteria like *L. plantarum* and *L. brevis* were isolated (protocol 2.2.9 as mentioned in chapter 2) amplified with primers LipLPF (5'-AACTTGCTTCGTCAACACGC-3') and LipLPR (5'-TGTTTGTGCATTCGCAAGGG-3') for *L. plantarum* and Lip LBF (5'ATGCTGAATGATACAACGAC3') and Lip LBR (5' TTAAATGAATTTTGCCGCTG 3') for *L. brevis.* Primers were designed using Gene runner and Primer blast software. As the gene sequences of these strains were not available in databases, the sequences that are already reported in Uniport (Universal protein resource), a database for proteins (**JDM1_0831** for *L. plantarum* and **LVIS_0232** for *L. brevis*) were taken and primers were designed.

5.2.2 Construction of pMALc5X expression vector

The forward and reverse primers specific to lipase gene were modified by incorporating the restriction sites for EcoR1 and XmnI. The reaction mixture for amplification were performed in a final volume of 25 µl containing

Buffer	2.5 µl
dNTPs (10 mM)	0.5 µl
Primers	0.5 µl
Taq DNA polymerase	1 µl
DNA	1 µl
Water	19.5 µl

The reaction was performed with the following program: denaturation at 95 ^{o}C for 3 min, followed by 35 cycles of 94 ^{o}C for 1 min, 53.4 ^{o}C for 30 s, 72 ^{o}C for 95 s, and then final extension for 72 ^{o}C for 5 min. The PCR product was detected on a 0.8% agarose electrophoretic gel. Gene fragments of 1000 base pairs for *L. plantarum* and 1400 base pairs for *L. brevis* were obtained as shown in Figure 5.1.

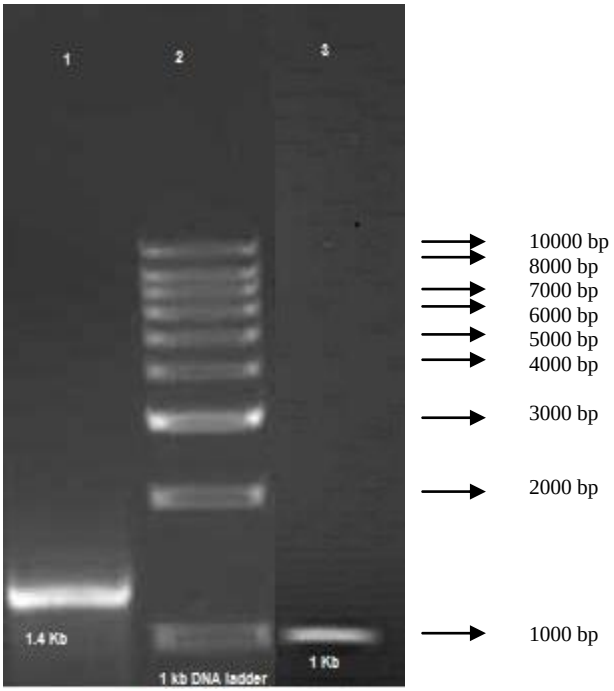


Figure 5.1: Lane 1 showing 1400 bp for *L. brevis*, lane 2 showing 1Kb step up DNA ladder, lane 3 showing 1000 bp for *L. plantarum*.

The gel was eluted with DNA gel Extraction kit (protocol 2.3.1 as mentioned in chapter 2). The purified DNA fragments were ligated into the cloning vector pMALc5X.

For insert and vector digestion the reaction mixture contains

For vector digestion and insert digestion (10 µl)

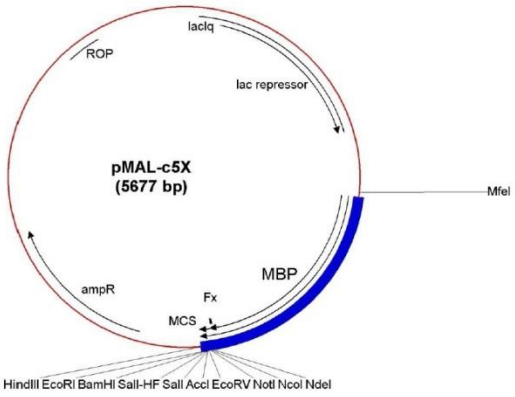
Vector or PCR product	3 µl
10X assay buffer	1 µl
Bam H1	2 µl
Xmn1	1 µl

Water	3 µl

For ligation (10 µl)

Digested vector	2 µl
Digested insert	4 µl
T_4 DNA ligase	2 µl
Water	2 µl

The schematic diagram and restriction sites of pMALc5X were shown in Figure 5.2.



pMAL-c5X Polylinker:

SacI XmnI

5´ *malE*...TCG AGC TCG (AAC)$_n$ AAT AAC AAT (AAC)$_n$ CTC GGG ATC GAG GGA AGG ATT TCA

NdeI NcoI NotI EcoRV SalI BamHI EcoRI SbfI

CAT ATG TCC ATG GGC GGC CGC GAT ATC GTC GAC GGA TCC GAA TTC CCT GCA GGT

AAT TAA ATA A...

Figure 5.2: Schematic diagram of the vector pMALc5X

The vector contains Maltose binding protein (MBP). Fusion of a target protein to MBP permits its one-step purification using amylose resin [225] as shown in Figure 5.3. Lipase gene was cloned into a pMAL vector downstream of the malE gene that encodes MBP. Upon induction, this system fuses the target protein sequence with a portion of MBP to create a fusion protein that is isolated using amylose affinity chromatography. Insertion of the lipase gene was confirmed by restriction analysis and sequencing. The expression vector

was transferred into host and the growth of bacterial cells and the induction of expression with IPTG were performed.

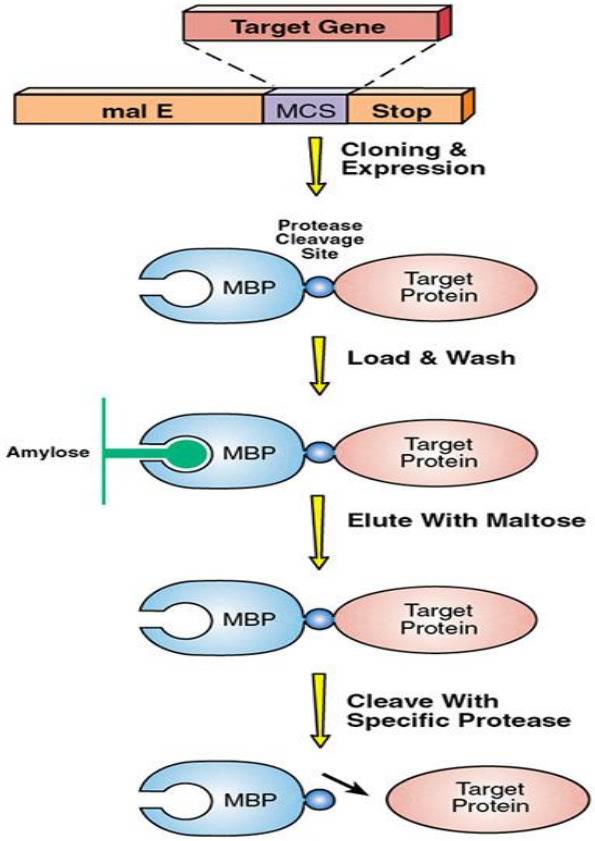


Figure 5.3: Fusion of target protein and purification using amylose resin

Source adapted from https://www.neb.com/applications/protein-expression-and-purification/coupled-protein-expression-and-purification/maltose-binding-protein

5.2.3 Expression and purification of the recombinant lipase:

Recombinant vector was then transformed into *E. coli* competent cells (protocol 2.3.2 as mentioned in chapter 2), and plated on LB (Luria Bertani) agar plates containing 100 µg/ml ampicillin. Cultures were transferred to 100 ml flasks containing LB medium and incubated at 37 ^{o}C until an O.D of 0.8 was reached. IPTG was added and incubated for 3-4 hrs with a final concentration of 1 mM. Cells were harvested by centrifugation (at 7830 xg, 10 min and 4 ^{o}C). The supernatant was discarded and the cells were resuspended in 25 ml of

column buffer (20 ml of 20 mM Tris-HCl, 11.7 gm of NaCl, 2.0 ml 0.5 M EDTA/lt). Then Amylose resin was poured in a 2.5 x 10 cm column. The column was washed with 5 volumes of column buffer. Diluted crude extract was loaded and washed with column buffer. The fusion protein was eluted with column buffer + 10 mM maltose. (The fusion protein usually starts to elute within the first 5 fractions). The protein was eluted twice by dialysis (20 mM Tris-HCl, 25 mM NaCl, pH 8.0).

5.2.4 Partial purification of native protein

The culture supernatant containing extracellular lipase obtained from fermented broth after optimization was treated with 0.4M $CaCl_2$ in order to precipitate fatty acids followed by centrifugation at 4 ^{o}C and 7830 xg for 30 min. The supernatant was collected in a glass beaker and to it chilled acetone was added slowly with continuous stirring, up to 70% (v/v) concentration andkept at 20 ^{o}C for 4 h to allow protein precipitation. The precipitates were then harvested by centrifugation at 4 ^{o}C and 7830 xg for 30 min. The pellet thus obtained was resuspended in 34 ml of 20 mM Tris-HCl buffer (pH 8.2) to allow the solubilization of proteins. Unsoluble proteins were then removed by centrifugation at 4 ^{o}C; 7830 xg for 30 min. Supernatant was subsequently subjected to dialysis overnight against the same buffer at 4 ^{o}C. The protein content and lipase activity were determined after each step.

5.2.5 DEAE (Diethyl amino ethyl) sepharose column chromatography:

5 ml of sepharose resin (Sigma) was packed into 10ml of glass column and kept overnight at 4 ^{o}C. The column was initially washed with distilled water twice followed by 50 mM Tris–HCl buffer (pH 8.2) containing 1mM phenylmethylsulfonyl fluoride (PMSF) thrice. After washing the crude enzyme was added to a DEAE-Sepharose column and elution was performed with a negative linear gradient of 0-1.0 M NaCl. Fractions showing lipase activity were pooled, concentrated by ultra-filtration and stored at -20 ^{o}C.

5.2.6 SDS-PAGE electrophoresis

The molecular mass of the lipase was determined by SDS-PAGE [226]. SDS-PAGE was carried out on 12% gel using SDS electrophoresis unit (GE Healthcare Life Sciences) following the Laemmli method. Molecular markers used were (Bangalore Genei protein marker medium range) of 14.3-97.4 KDa. Proteins in the gel were stained with Coomassie brilliant blue R-250. A polypeptide with a molecular weight of about 65 KDa for *L. plantarum* and 26 KDa for *L. brevis* were obtained as shown in Figure 5.4.

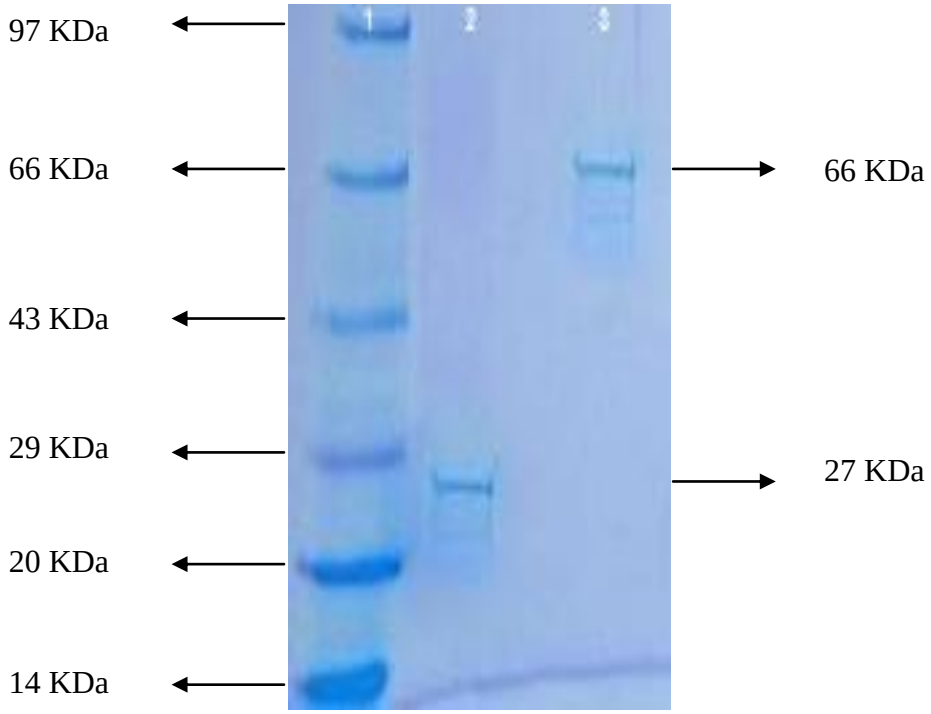


Figure 5.4 SDS: Lane 1 showing protein marker, Lane 2 and 3 are the purified fractions of the expressed enzyme showing 27 KDa and 66 KDa of *L. brevis* and *L. plantarum*.

5.2.7 Determination of lipase activity

The lipase assay was performed spectrophotometrically using *p*-nitrophenyl palmitate as substrate. The assay mixture contained 2.5 ml of 420 µm *p*-nitrophenyl palmitate, 2.5 ml of 0.1 M Tris – HCl (pH-8.2) and 1 ml of enzyme solution. It was incubated in water bath at 37 ^{o}C for 10 min and p-nitrophenol was liberated from *p*-nitrophenyl

palmitate by lipase mediated hydrolysis imparting a yellow color to the reaction mixture. After incubation, the absorbance was measured at 410 nm [227]. One unit (U) of lipase was defined as the amount of enzyme that liberates one micromole of *p*-nitrophenol per minute under the assay conditions [228].

The specific activity of lipase before expression was 12.5 U/mg (enzyme activity was 35 U/ml) for *L. brevis* and 13.2 U/mg (enzyme activity 37 U/ml) for *L. plantarum*. The expressed lipase of *L. brevis* showed 3 fold increase in specific activity of 36.42 U/mg (enzyme activity 102 U/ml) after expression and similarly the expressed lipase of *L. plantarum* showed 3.3 fold increase in specific activity of 42.8 U/mg (enzyme activity 110 U/ml) after expression which showed a significant level of expression as shown in the table 5.1.

Table 5.1: Enzyme activity and specific activity of the lipase enzyme

Organism	Before optimization		After optimization		After expression	
	Enzyme activity (U/ml)	Specific activity (U/mg)	Enzyme activity (U/ml)	Specific activity (U/mg)	Enzyme activity (U/ml)	Specific activity (U/mg)
L. brevis	14±0.05 U/ml	5 ±0.052 U/mg	35±0.10 U/ml	12.5±0.05 U/mg	102±0.1 U/ml	36.2 ±0.05 U/mg
L. plantarum	16±0.05 U/ml	6 ±0.03 U/mg	37±0.15 U/ml	13.2 ±0.05 U/mg	110 ±0.42 U/ml	42.8 ±0.05 U/mg

Data was mean standard deviation of 3 experiments ($p<0.05$) which showed significant data

Specific activity is the number of enzyme in units/ml divided by concentration of protein in mg/ml. Therefore the unit of specific activity is U/mg.

Table 5.2: Lipase purification by various methods

Purification steps	Volume (ml)	Protein (mg/ml)	Enzyme activity (U/ml)		Lipase recovery (%)
			L. brevis	*L. plantarum*	
Crude extract	50	6.4	35±0.11	37±0.15	100
Acetone treatment	5	3.9	42±0.15	46±0.15	80
DEAE-sepharose Column	15	3.2	64.26±0.16	79±0.11	40
Amylose column affinity chromatography	10	2.8	102±0.1	110±0.5	20

Data was obtained from 3 experiments with ($p<0.05$)

5.2.8 Sequence analysis

A comparative study of the sequences of *L. plantarum* and *L. brevis* was done by pair wise sequence alignment to check the similarity between the lipase genes. Pair wise sequence alignment obtained with EMBOSS-Needle program of the extracellular lipases from *L. brevis* and *L. plantarum* showed only 23.2% similarity.

```
Q03TS4_LACBA      1 MSLRGKWLLFKTQHSQLKQQIQQAFLQPSRQNRAVIPEKFATSIVTKERQ     50

C6VN50_LACPJ      1 --------------------------------------------------      0

Q03TS4_LACBA     51 IFGGRLLTAATGAPLPQHVLLFHGGAYTMQGIDSHRELMETLIQ------     94
                    ||.|  .::|..|..|
C6VN50_LACPJ      1 ----------------------------MQVI--KQKLTATCAQLTGYLH     20

Q03TS4_LACBA     95 -------QANLRVTYV----DYPLVPEATVDE-TVVFAMNAYDYLRAQY-    131
                    |.||....:    .|..:|.|..:. .:.||.:.|.....:|
C6VN50_LACPJ     21 QPDTNAHQTNLPAIIIVPGGSYTHIPVAQAESLAMAFAGHGYQAFYLEYT     70

Q03TS4_LACBA    132 --PDDQFFLMGDSAGGGLALTLLQQLAK--HQEPM---PAG--------T    166
                    .|.|...:......|.|:.||:|.|.  |.:|.   |||        .
C6VN50_LACPJ     71 LLTDQQPLGLAPVLDLGRAVNLLRQHAAEWHIDPQQITPAGFSVGGHIVA    120

Q03TS4_LACBA    167 ILVSPW-----TDLSMMNPDLTLAAKRDPYLTLATLKK----IGFDYAGD    207
                    :....|    |:|:              :|.|.||.    :|:
C6VN50_LACPJ    121 LYNDYWATRVATELN----------VTPAMLKPNNVVLGY-----    150

Q03TS4_LACBA    208 HPVTDPVVSPIYG------------------------NFDNLGPVLLYYG    233
                    ||:||:.|                     |.||....:....
C6VN50_LACPJ    151 -----PVISPLLGFPKDDATLATWTPTPNELAADQHVNSDNQPTFIWTTA    195

Q03TS4_LACBA    234 TNELLAADDERLLQKLQAASGTPFQAHQMPSMLHDYIL------WRKLPE    277
                    .:.::.|.:.........|.:...|::.|......|...|     |:  |:
C6VN50_LACPJ    196 DDPIVPATNTLAYATALATAKIPYELHVFKHGPHGLALANAQTAWK--PD    243

Q03TS4_LACBA    278 TKQT------------LAEIKQFILTGEL    294
                    ..|.          ||:.:
C6VN50_LACPJ    244 ANQPHVAHWLTLALEWLADNR--------    264
```

5.3 Discussion

Heterologous expression is one of the promising strategies to increase the production of enzyme at industrial level. In the present study heterologous expression and purification of native and expressed lipase enzyme was studied. The DNA was successfully amplified by PCR. Figure 5.1 showed the gene fragments of 1000 base pairs for *L. plantarum* and 1400 base pairs for *L. brevis.* The amplified gene was cloned in vector pMALc5X and expressed in *E. coli* BL21 (DE3) pLysS. A successful transformation will always be beneficial for increased production of enzyme at industrial level.

The final purity of the enzymes from *L. brevis* and *L. plantarum* was observed by DEAE-Sephadose column chromatography for native enzyme and Amylose column for expressed enzymes. The molecular weights were determined by SDS-PAGE. While determining the molecular weight of the enzymes by SDS-PAGE electrophoresis it was found that the enzyme from *L. brevis* showed a molecular weight of 27 KDa and the lipase enzyme from *L. plantarum* showed a molecular weight of 66 KDa.

When compared to the enzyme activity of 64.26 U/ml of native enzyme, the expressed purified enzyme showed the highest activity of 102 U/ml which proved that the recombinant enzyme was more attractive for large scale production and wide-range of industrial applications than native enzyme.

A pair sequence alignment was done to see the similarity of lipase genes between the sequences of *L. plantarum* and *L. brevis* and found that they are only 23.2% similar.

5.4 Conclusion

This study established the heterologous expression potential of lipase genes from *L. brevis* and *L. plantarum*. The novel lipase produced as a result of heterologous expression from the two bacterial strains showed a threefold increase in its activity when compared to the native form.

Immobilization and kinetic study of expressed and immobilized lipase

6.1 Introduction

Enzymes are selectively specific biomolecules that aid in industrial biotransformation reaction potentially substituting the chemical catalysts [229-230]. Despite their advantages, certain properties like difficulty in enzyme recovery for their reutilization and intolerance towards extreme pH and thermal stability make them inadequate for industrial applications [231]. Hence before using enzymes as biocatalysts, they are significantly improved by different techniques like molecular biology, immobilization and post immobilization techniques and reactor engineering techniques where immobilization method was significant.

Enzymes are immobilized on different supports to increase the properties like reusability, self-life, stability and recovery [232]. The techniques used to immobilize is an important factor for large scale use in industry. Immobilization has several advantages like production of high purity products, reuse of enzymes, easy separation of products, high pH and thermal stability [233]. Lipases are immobilized on different supports with different immobilization techniques [234-237]. Different methods like adsorption, entrapment, covalent binding, cross linking were used to immobilize the enzyme on solid support.

Lipase from *Pseudomonas aeruginosa* BBRC-10036 was immobilized into calcium alginate beads by entrapment method [238]. The optimum conditions for immobilization were studied using Response surface methodology and optimum activity of 2.64 U/g was obtained using immobilization.Similarly a thermostable lipase enzyme produced from thermophilic *Bacillus* sp was immobilized on silica and HP-20 beads by cross linking method that increased the thermostability of the enzyme [239]. Sodium alginate immobilized lipase

from *Bacillus cereus* MS6 showed an increased activity of 550 U/ml compared to free enzyme [240].

Immobilized lipase from *Bacillus smithii* BTMS11 was optimized using one factor at a time method and after optimization it was observed that the immobilized lipase could be applied in effluent water treatment [241]. A lipase enzyme from *Azospirillum* sp. PRD1 was immobilized on chitosan alginate. The activity of beads were measured by titrimetric method and found that the activity was 90 U/ml [242].

The rate of activation and inactivation of enzymes and their functional efficiency together constitutes the kinetic data of an enzyme. The determination of K_m and V_{max} values of enzyme system was necessary to determine the kinetic potentiality of enzymes and their specificity. Reaction rates are often heavily influenced principally by conditions such as substrate, inhibitor, pH, and temperature. These kinetic factors must be of great importance in controlling the rates of enzyme-catalyzed reaction.

Lipase from *Pencillium chrysogenum* SNP5 was immobilized on silica gel and polyacrylamide beads and different kinetic properties like pH stability, K_m and V_{max} were studied. High thermostability, specificity and wide range of pH were observed after immobilization [243]. Acidic lipase derived from *Pseudomonas gesardii* was immobilized in mesoporous activated carbon (MAC_{400}). The thermal stability and reusability of immobilized lipase was found to be better than native enzyme [244].

In this chapter, a study of the immobilization of lipase by calcium alginate and kinetics of expressed and immobilized lipase enzyme from probiotic lactic acid bacteria like *L. brevis* and *L. plantarum* has been reported along with its parametric behaviour.

6.2 Methodology

6.2.1 Source of the enzyme

L. plantarum and *L. brevis* were used as sources of lipase enzyme.

6.2.2 Preparation of sodium alginate gel (3%)

3 g of sodium alginate was dissolved in 100 ml of distilled water and mixed thoroughly to make a transparent gel and left undisturbed for 30 min to remove the air bubbles. After 30 min of incubation the gel was used for entrapment of the enzyme.

6.2.3 Determination of lipase activity

The lipase assay was performed spectrophotometrically using *p*-nitrophenyl palmitate as substrate. The assay mixture contained 2.5 ml of 420 µm *p*-nitrophenyl palmitate, 2.5 ml of 0.1 M Tris-HCl (pH-8.2) and 1 ml of enzyme solution. It was incubated in water bath at 37 ^{o}C for 10 min. Then *p*-nitrophenol was liberated from *p*-nitrophenyl palmitate by lipase mediated hydrolysis imparting a yellow color to the reaction mixture. After incubation, the absorbance was measured at 410 nm. One unit (U) of lipase was defined as the amount of enzyme that liberates one micromole of *p*-nitro phenol per minute under the assay conditions [245].

6.2.4 Preparation of immobilized enzyme using sodium alginate

Sodium alginate gel was prepared by suspending 3 g of sodium alginate in 100 ml distilled water [246], stirred for 5 min and incubated for 30 min. To this suspension 0.5 ml of lipase enzyme was added and mixed thoroughly. Beads of uniform size were formed by dripping the solution in freshly prepared 250 ml of chilled 0.2 M calcium chloride. Beads were left in calcium chloride for 3-4 hrs and then resuspended in Tris-HCl buffer (8.2). The beads were washed several times with the buffer to remove unbound enyme. The enzyme activity of the beads was calculated. The units of enzyme entrapped in a gel were calculated

by subtracting the units of non-entrapped enzyme from total units of the enzyme [247]. The total units of enzyme were 102 U/ml for *L. brevis* and 110 U/ml for *L. plantarum.*

6.3 Results

6.3.1 Effect of pH on enzyme activity

To determine the effect of pH on the activity of expressed and immobilized lipase, both were incubated in differentbuffers with pH 5-5.5 (acetate); pH 6-7 (phosphate) and pH 8-8.5 (Tris-HCl) at 4 ^{o}C for 24 hrs.

The expressed lipase enzyme was active at pH 7 for *L. brevis* and pH 6 for *L. plantarum* and the immobilized lipase enzyme was active at pH 7 for *L. brevis* and pH 6.5 for *L. plantarum* as shown in Figure 6.1. Further increase in pH caused rapid decrease in enzyme activity.

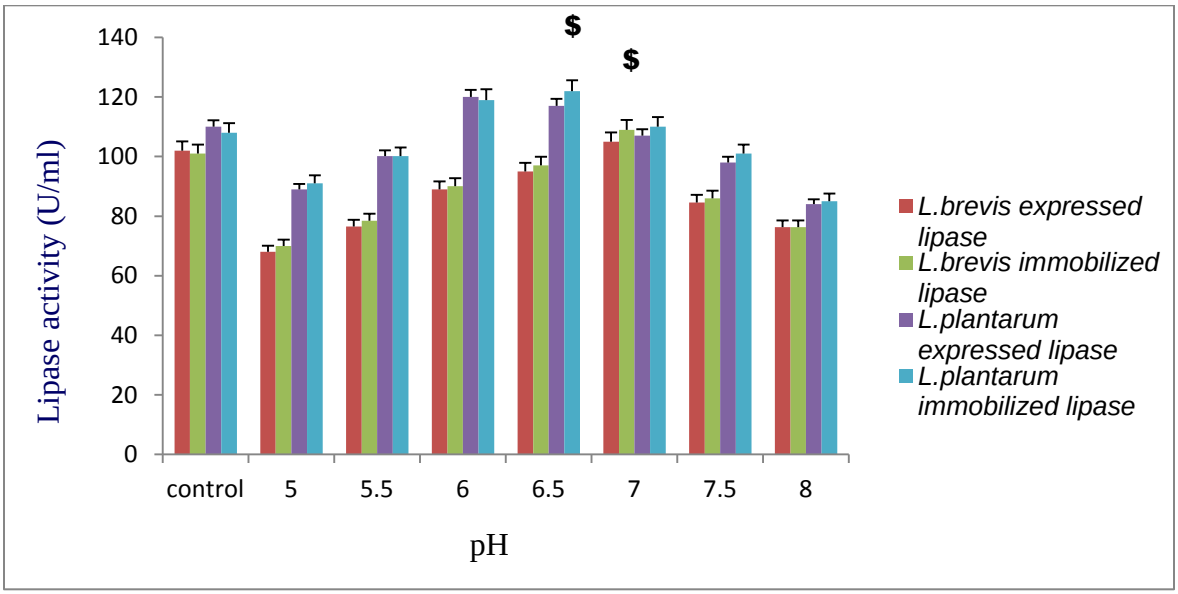


Figure 6.1: Effect of pH on activity of expressed and immobilized lipase

Data was mean standard deviation of 9 replicates from 3 experiments ($p \leq 0.05$). $^{\$}$ represents maximum enzyme activity obtained at pH 6.5 for *L. plantarum* and pH 7 for *L. brevis*.

6.3.2 Effect of temperature on enzyme activity

A change in temperature may affect the stability of the enzyme leading to permanent loss of enzyme activity. Therefore to evaluate the effect of temperature on activity of expressed and immobilized lipase, the enzyme was incubated at different temperatures ranging from 25 to 50 ^{o}C in Tris-HCl buffer with pH 8.0 at an interval of 5 ^{o}C.

The optimum activity of the expressed enzyme was found to be 37 ^{o}C for *L. brevis* and 40 ^{o}C for *L. plantarum*. Similarly the optimum activity of the immobilized enzyme was found to be 40 ^{o}C for *L. brevis* and 45 ^{o}C for *L. plantarum* as shown in Figure 6.2.

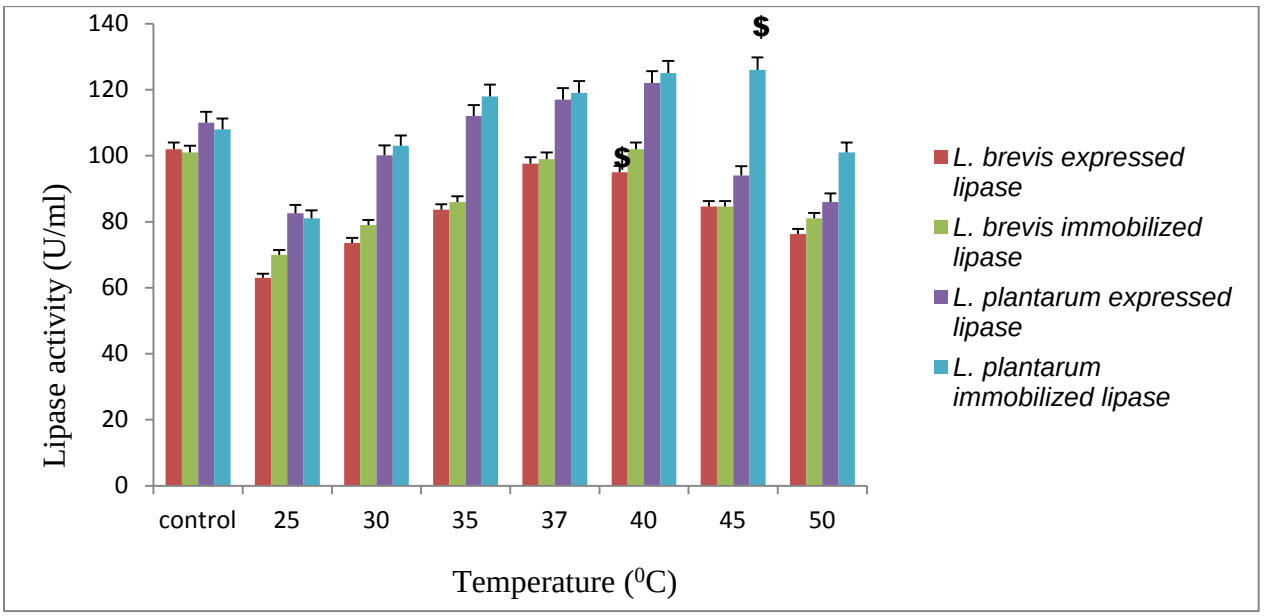


Figure 6.2: Effect of temperature on expressed and immobilized enzyme activity

Data was mean standard deviation of 9 replicates from 3 experiments ($p \leq 0.05$). $^{\$}$ represents maximum enzyme activity obtained at temperature 45 ^{o}C for *L. plantarum* and 40 ^{o}C for *L. brevis*.

6.3.3 Effect of metal ions and inhibitors on enzyme activity

The effect of different metal ions like K^{+}, Na^{+}, Cu^{2+}, $Fe^{2+}$and Mg^{2+} and inhibitors like EDTA (ethylenediaminetetraaceticacid) and SDS (sodium dodecyl sulphate) were studied on

the activity of enzyme by incubating both expressed and immobilized enzyme with specified ion (at 1 mM) containing buffer (Tris–HCl with pH 8.0)and the activity was measured using *p*-nitrophenyl palmitate as substrate.

The activity of the both expressed and immobilized lipase enzyme was enhanced by Mg^{2+} ions for *L. brevis* and *L. plantarum* where as EDTA and SDS decreased the activity of the enzyme as shown in Figure 6.3.

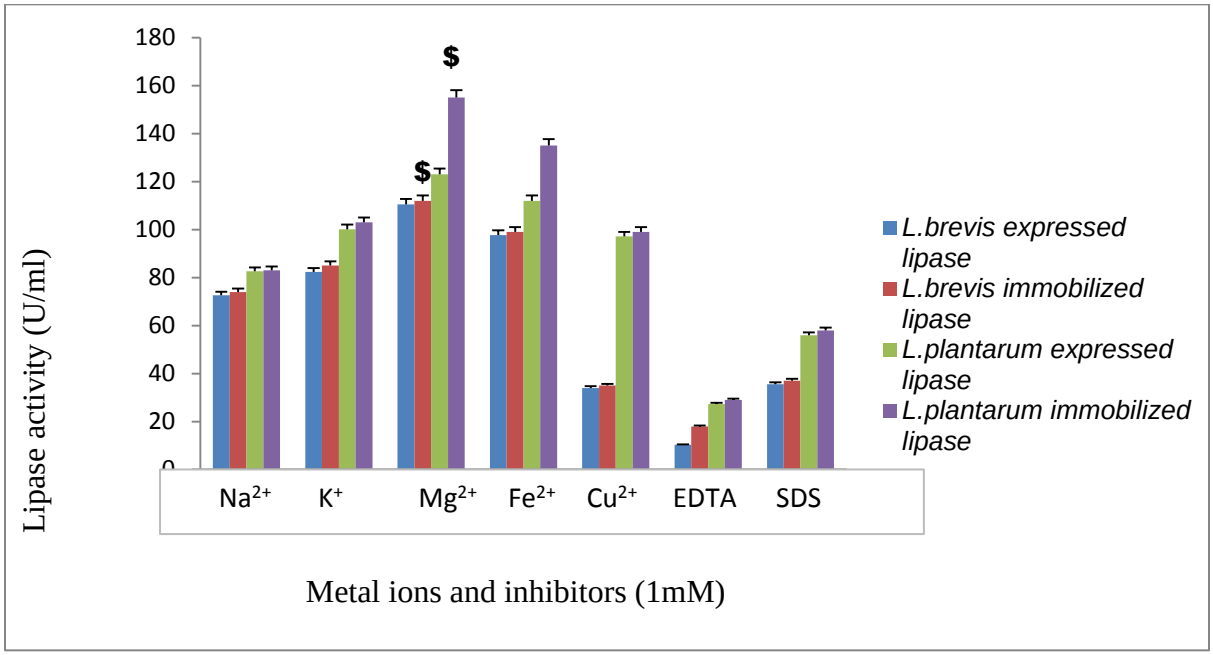


Figure 6.3: Effect of metal ions and inhibitors on enzyme activity of expressed and immobilized lipase

Data was mean standard deviation of 9 replicates from 3 experiments. ($p \leq 0.05$). $^{\$}$ represents maximum enzyme activity obtained with Mg^{2+} for *L. plantarum* and *L. brevis*.

6.3.4 Kinetic study

The Michaelis-Menten kinetic parameters V_{max} and K_m values of lipase were calculated using *p*NPP (*p*-nitrophenyl palmitate) as substrate at concentrations ranging from 0.5 mM to 2.5 mM at an interval of 0.5 mM.

The kinetic parameters V_{max} and K_m were determined from Lineweaver-Burk plots. The V_{max} and K_m values of the enzyme for *L. brevis* were 1.28 µmol/mg/min and 0.44 mM as shown in the Figure 6.4.

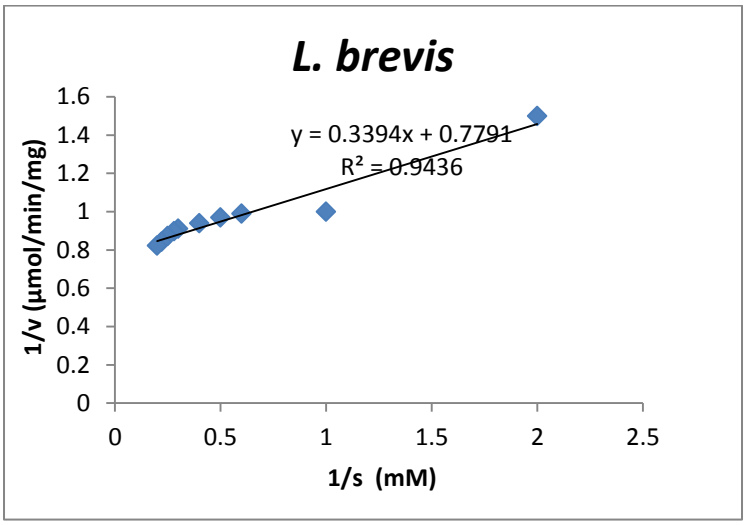


Figure 6.4: V_{max} and K_m values of lipase from *L. brevis*

Similarly the V_{max} and K_m values of the enzyme for *L. plantarum* were 1.44 µmol/mg/min and 0.426 mM respectively as shown in Figure 6.5.

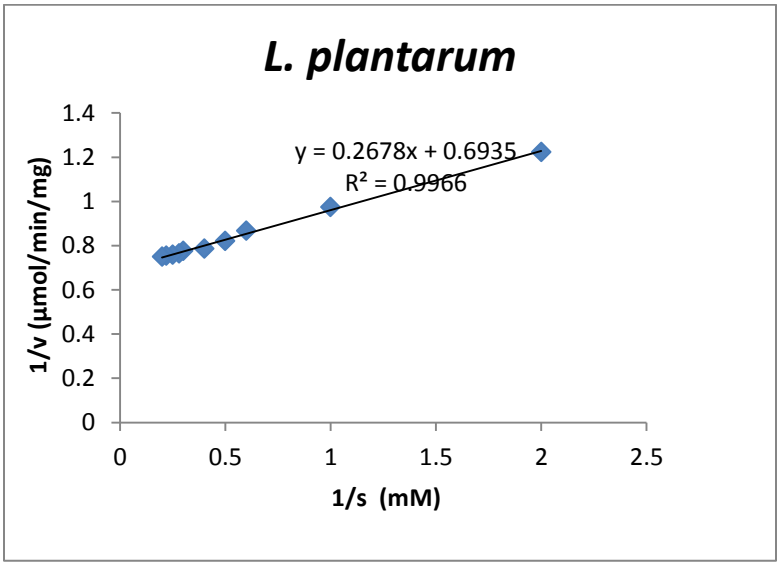


Figure 6.5: V_{max} and K_m values of lipase from *L. plantarum*

The V_{max} and K_m values of the immobilized enzyme for *L. brevis* were 1.33 µmol/mg/min and 0.39 mM as shown in the Figure 6.6.

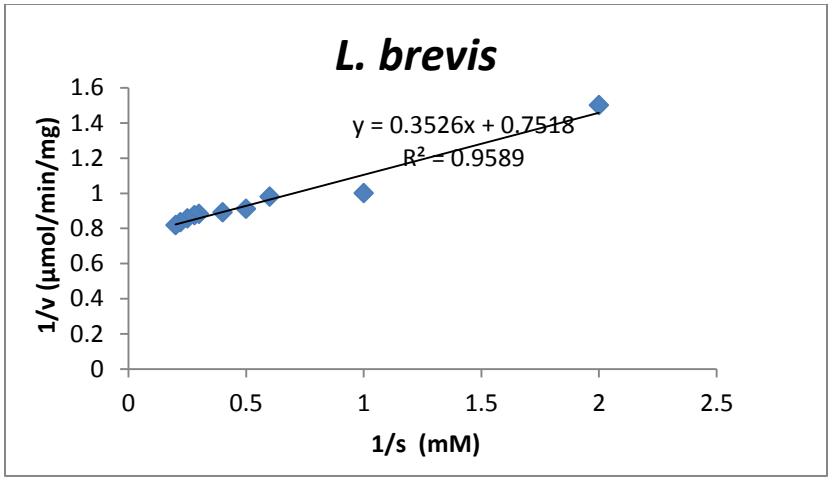


Figure 6.6: V_{max} and K_m values of immobilized lipase of *L. brevis*

Similarly the V_{max} and K_m values of the enzyme for *L. plantarum* were 1.47 µmol/mg/min and 0.37 mM respectively as shown in Figure 6.7

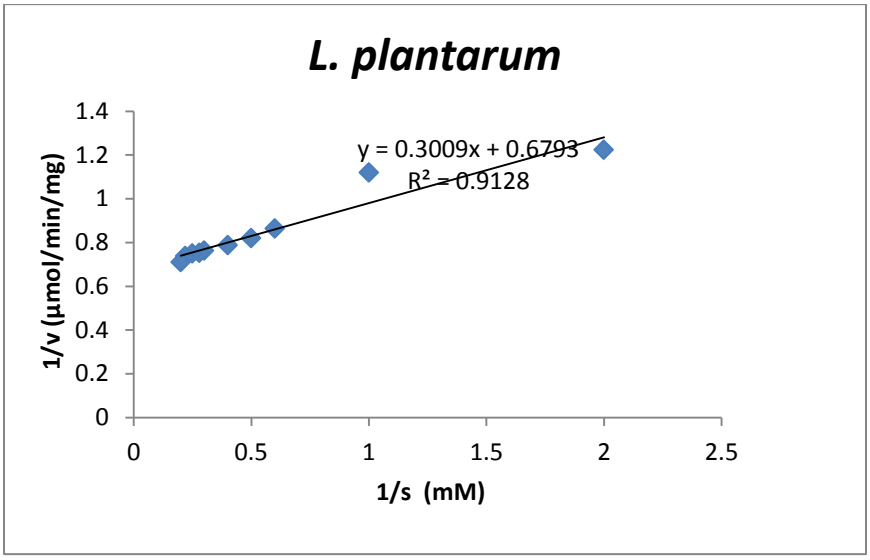


Figure 6.7: V_{max} and K_m values of immobilized lipase of *L. plantarum*

The table given below shows a comparative study of kinetic properties of expressed and immobilized enzyme.

Table 6.1: Comparative study of kinetic properties of expressed and immobilized lipases from *L. brevis* and *L. plantarum.*

Parameter	Expressed enzyme		Immobilized enzyme	
	L. brevis	*L. plantarum*	*L. brevis*	*L. plantarum*
pH	7	6	7	6.5
Temperature	37 ^{o}C	40 ^{o}C	40 ^{o}C	45 ^{o}C
Activators and Inhibitors	Mg^{2+} EDTA, SDS	Mg^{2+} EDTA, SDS	Mg^{2+} EDTA, SDS	Mg^{2+} EDTA, SDS
V_{max}	1.28 µmol/mg/min	1.44 µmol/mg/min	1.33 µmol/mg/min	1.47 µmol/mg/min
K_m	0.44 mM	0.426 mM	0.39 mM	0.37 mM

6.4 Discussion

Figure 6.1 showed the effect of pH on expressed and immobilized lipase of *L. brevis* and *L. plantarum.* From the figure 6.1 it was evident that there was shift in pH from expressed enzyme to immobilized enzyme of *L. plantarum* from pH 6 to 6.5. This shift in optimum pH could be due to the change in acidic and basic amino acid side chain ionization in the microenvironment around the lipase active site. But for *L.brevis* no change in pH was observed. The reason might be due to low activity of lipase enzyme of *L.brevis* compared to *L. plantarum* and less ability of the enzyme to form product.

Figure 6.2 showed effect of temperature on expressed and immobilized lipase where temperature variation was seen in expressed and immobilized enzyme which might be due to hydrophilicity of alginate gel and hydrophobicity and flexibility of enzyme.

Figure 6.3 showed the effect of metal ions on expressed and immobilized lipase, it was evident that much variation was not observed for most of the metals and activity of expressed and immobilized enzyme was only enhanced by Mg^{2+} ions. The reason could be

attributed to stabilization of the active site of the enzyme, presence of EDTA and SDS decreased the activity of the enzyme, as chelating agent EDTA and anionic detergent SDS might have caused conformational changes.

Figures 6.4-6.7 showed the K_m and V_{max} values of expressed enzyme and immobilized enzyme. From the figures it was observed that the K_m value of expressed enzyme was less compared to immobilized enzyme which showed that enzyme substrate complex of expressed enzyme was more stable than that of immobilized enzyme. The increase in K_m value might be due to less stability of enzyme-substrate complex. The V_{max} value was more for expressed enzyme and less for immobilized which might be due to conformational changes (change in shape of macromolecule) in the enzyme that occurs due to environmental factors and other factors like pH, voltage, ion concentration etc.

The comparative study of kinetic properties of expressed and immobilized lipase is shown in Table 6.1. It is evident that immobilization using sodium alginate can be extrapolated for large scale production of lipase.

6.5 Conclusion

Enzymes can replace the conventional catalysts in industrial application. The property of immobilizing enzymes on different materials can meet the required performance in enzyme catalysis. Hence the above study was shown to be a promising strategy for obtaining an active and stable lipase through successful immobilization using sodium alginate bead by entrapment method. The immobilized lipase can bind to a wide range of substrates and can be reused for number of reactions for production of different industrial products.

Lipase mediated ester synthesis and their industrial applications

This chapter includes lipase mediated ester synthesis and industrial application of lipases and esters in different industries.

7.1 Introduction

Lipases are triaclyglycerolacylhydrolases (EC 3.1.1.3) that catalyze the hydrolysis of triaclyglycerol to glycerol and fatty acids [248]. They catalyze different reactions like esterification, transesterification, hydrolysis, aminolysis, acidolysis, regioselective acylation of glycerols resulting in synthesis of peptides, esters, amides and other chemicals [249]. Lipases are widely used in different industries such as food, detergent, pharmaceutical, leather textile, cosmetic, biodiesel production, degreasing, flavor and paper industries [250]. Lipases are considered as biotechnologically important mainly due to versatility of their applied properties, substrate specificity and ease of mass production.

These enzymes are used as additives in detergents in removing stains. These are biodegradable and environmental friendly compared to chemical additives [251]. Lipases are widely used in synthesis of short chain fatty acid esters which are used as flavoring agents and long chain fatty acids which are used as biofuel [252]. In medical field lipases are considered to be important biomarkers for diseases like acute pancreatitis and exocrine pancreatic insufficiency [253]. They also hydrolyze the pith from pulp which makes it widely used in pulp and industry [254].

Transesterification mediated by immobilized lipase from *Candida cylindracea* synthesized highly stable short chain fatty acid esters like ethylpropionate [255]. Glucose esters synthesized by immobilized *Candida* spare widely used in food industry as additives [256]. The immobilized lipase from *Candida antarctica* was used to synthesize adipate ester

by esterification of adipic acid and oleyl alcohol. The conditions for the synthesis were optimized using RSM method which resulted in very high yield of ester [257].

Thermotolerant, alkalophilic, synthetic *poly* (MAc-*co*-DMA-*cl*-MBAm) hydrogel immobilized lipase from *Bacillus coagulans* MTCC-6375 was used in esterification of methanol and 2-propanol for production of esters like methyl acetate and 2-propyl acetate [258]. Transesterification mediated by lipase from papaya latex and rice bran with methanol and palm oil resulted in synthesis of fatty acid methyl esters (FAME). The optimum conditions using RSM resulted in increased yield of such esters [259].

In this chapter some industrial applications of the lipase from *L. brevis* and *L. plantarum* and the esters synthesized from such a lipase is discussed

7.2 Methodology

7.2.1 Application of expressed lipase in fat degradation

Generally lipases catalyze the degradation of fat, oils and grease [260-262]. This property allows them to be used in treating lipid containing environment. Hence 10 gm of adipose tissue (fat) from chicken was weighed and autoclaved. 0.5 ml of enzyme was added to the tissue and incubated at 37 ^{o}C. A control was also kept where no enzyme was added and for every 12 hrs, the weight was taken to check the amount of degradation.

7.2.2 Application of lipase in ester synthesis

Short chain fatty acid esters have high importance in food industry. For this study all starting materials were purchased from Aldrich (India) and used directly without further purification. Esterification reaction was carried out in glass vials containing the solvent Tetrahydrofuran (THF) varying from (500-1000 µl) in vinyl acetate (varying from 100-500 µl) with different substrates (ranging from 50-200 mg) like 2-hydroxyl benzyl alcohol (2-HB), 3-hydroxyl benzyl alcohol (3-HB) and 4-hydroxyl benzyl alcohol, Triazole (GTRI). Reaction was initiated by addition of immobilized *Lactobacillus plantarum* lipase (50-

200mg). Samples were placed at different time intervals (varying from 24 -72 hrs) in an orbital shaker at different temperatures (4 ^{o}C, 37 ^{o}C, 60 ^{o}C) and rpm (100-300) along with the respective controls without immobilized lipase. Solvents were dried using standard methods and distilled before use. Visualization on TLC was achieved by use of UV light (at 254 nm). Column chromatography was performed for purification on silica gel (100–200 mesh, SRL, India) using ethyl acetate and hexane as elutant. The samples were analysed by IR (infrared spectroscopy) and NMR (Nuclear magnetic resonance spectroscopy).

^{1}H NMR (300 MHz and 400 MHz) and ^{13}C (75 MHz and 100 MHz) spectra were recorded in $CDCl_3$ and DMSO-d_6 solution with TMS as internal standard. IR spectra were recorded on KBr plates on Jasco FT/IR - 4200 instrument.

7.3 Results

7.3.1 Application of expressed lipase in fat degradation

The degradation of meat was obtained by taking weights and measuring enzyme activity by using *p*-nitrophenyl palmitate as substrate for every 12 hrs as shown in Table 7.1. Complete degradation in this study was observed at 96 hrs for *L.brevis* and 72 hrs for *L.plantarum* and lipase from co-culture showed complete degradation in 48 hrs.

Table 7.1: Showing the degradation of meat at different time periods

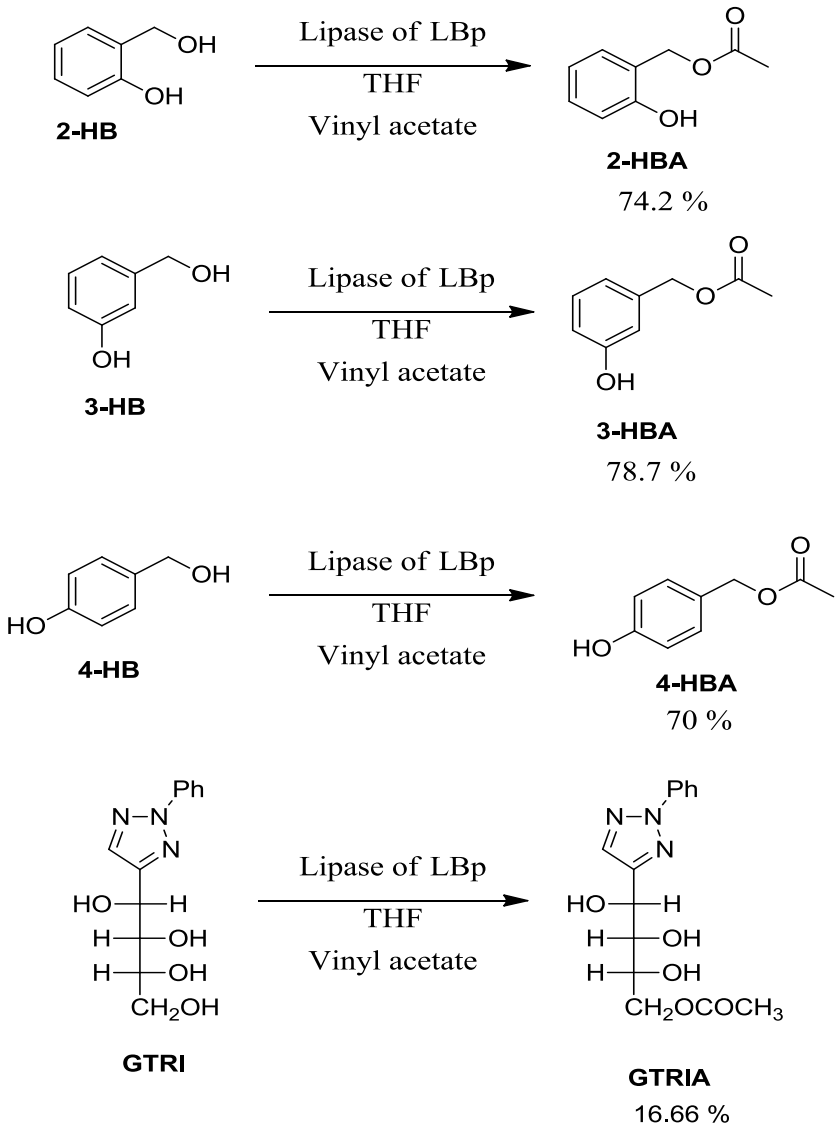
Organism	Initial weight (in gms)	Enzyme activity (U/ml)	24 hrs (weight in gms)	Enzyme activity (U/ml)	48 hrs (weight in gms)	Enzyme activity (U/ml)	72 hrs (weight in gms)	Enzyme activity (U/ml)	96 hrs (weight in gms)	Enzyme activity (U/ml)
Lipase from L. brevis	10.7 ±0.02	51± 0.32 U/ml	9.58 ±0.02	49±0.40 U/ml	6.9 ±0.03	44 ±0.15 U/ml	2.4±0.02	41 ±0.26 U/ml	Complete degradation	38 ±0.15 U/ml
Lipase from L. plantarum	10.7 ±0.01	55 ± 0.251 U/ml	8.2 ±0.02	53±0.36 U/ml	4.8 ±0.02	51 ±0.20 U/ml	Complete degradation	49± 0.25 U/ml	-	-
Lipase from Co-culture	10.7 ±0.02 U/ml	106± 0.30 U/ml	6.4 ±0.01	104±0.15 U/ml	Complete degradation	100 ±0.3 U/ml	-	-	-	-

Data is mean standard deviation of 9 replicates from three experiments and $p \leq 0.05$ showed significant data with control.

7.3.2 Application of lipase in ester synthesis

After the optimization studies mentioned in section 7.2.2, the short chain fatty acid esters were obtained with 900 µl of the solvent tetrahydrofuran (THF) in 100 µl (1.084 mmol) vinyl acetate with 50 mg of different substrates (1.2 mmol of 2, 3, 4-hydroxybenzylyalcohol, 0.566 mmol of triazole) and 150 mg of enzyme at 37 ^{o}C at 110 rpm with 60 hrs of incubation period as shown in Scheme 1.

Schematic representation of the reaction



The analysed samples with NMR were confirmed to be 2-hydroxybenzyl acetate, 3-hydroxybenzyl acetate, 4-hydroxybenzyl acetate and Triazole acetate.

2-hydroxybenzyl acetate (2-HBA)

IR Wavenumber [cm^{-1}] 3401.82, 2924.52, 1793.47, 1706.69, 1593.88, 1503.24, 1455.03, 1377.89, 1256.4, 1095.37, 1039.44, 928.557, 822.491, 754.031, 609.396, 528.4, 434.869.

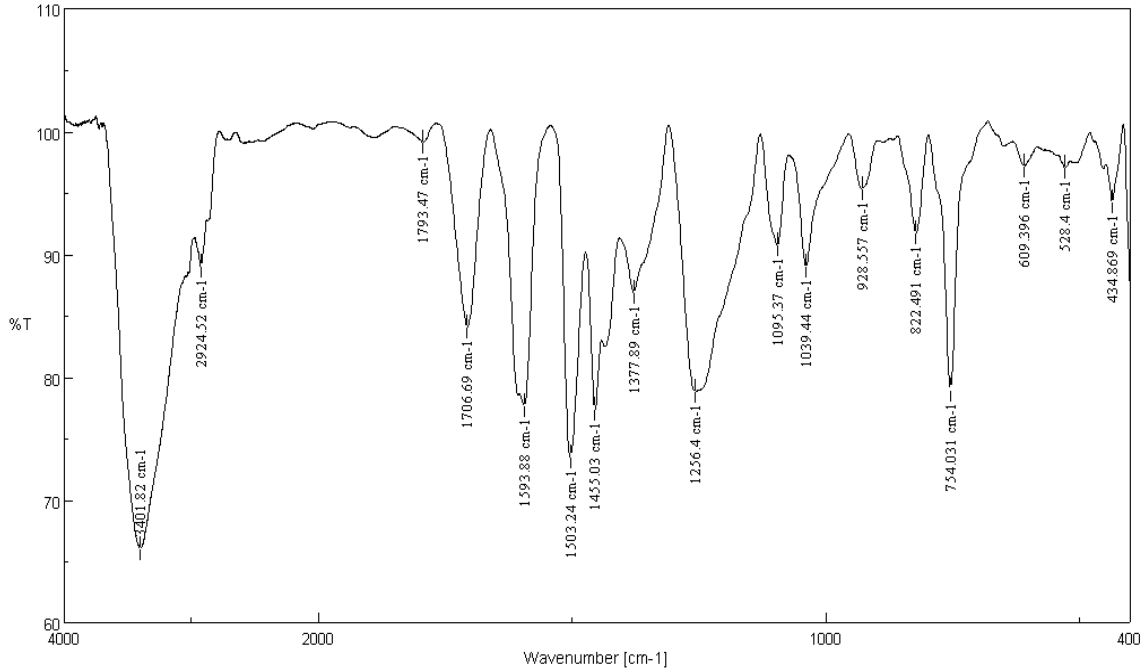


Figure 7.1a: IR plot showing 2-hydroxybenzyl acetate

^{1}H NMR (300 MHz, $CDCl_3$) δ 2.13 (s, 3H), 5.14 (s, 2H), 6.94 (dd, *J* = 13.4, 7.5 Hz, 2H), 7.30 (t, *J* = 7.4 Hz, 2H), 7.82 (s, 1H).

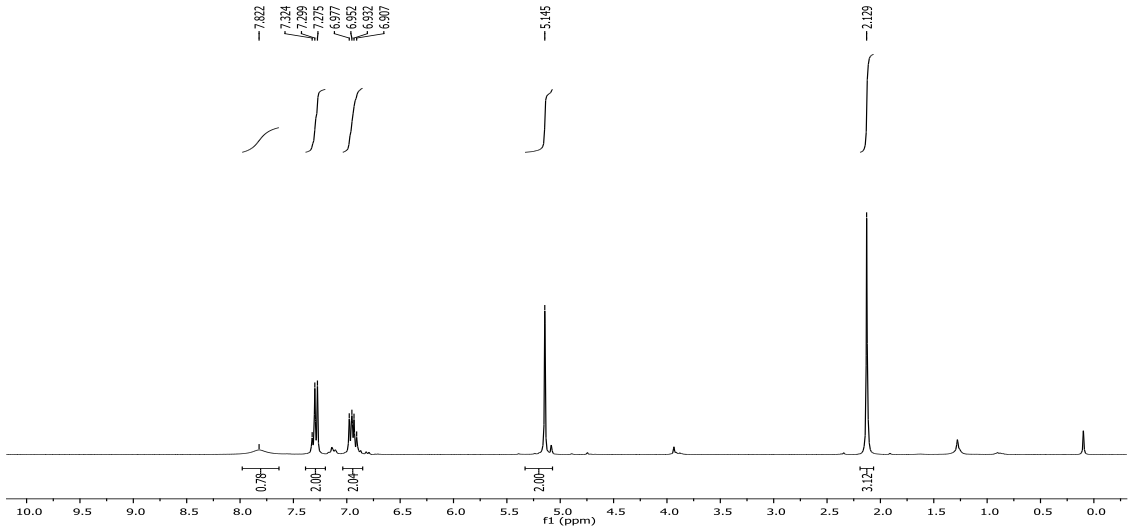


Figure 7.1b: ^{1}H NMR plot showing 2-hydroxybenzyl acetate

^{13}C NMR (75 MHz, $CDCl_3$) δ 20.95, 63.31, 117.81, 120.60, 121.65, 131.20, 132.22, 155.50, 173.80.

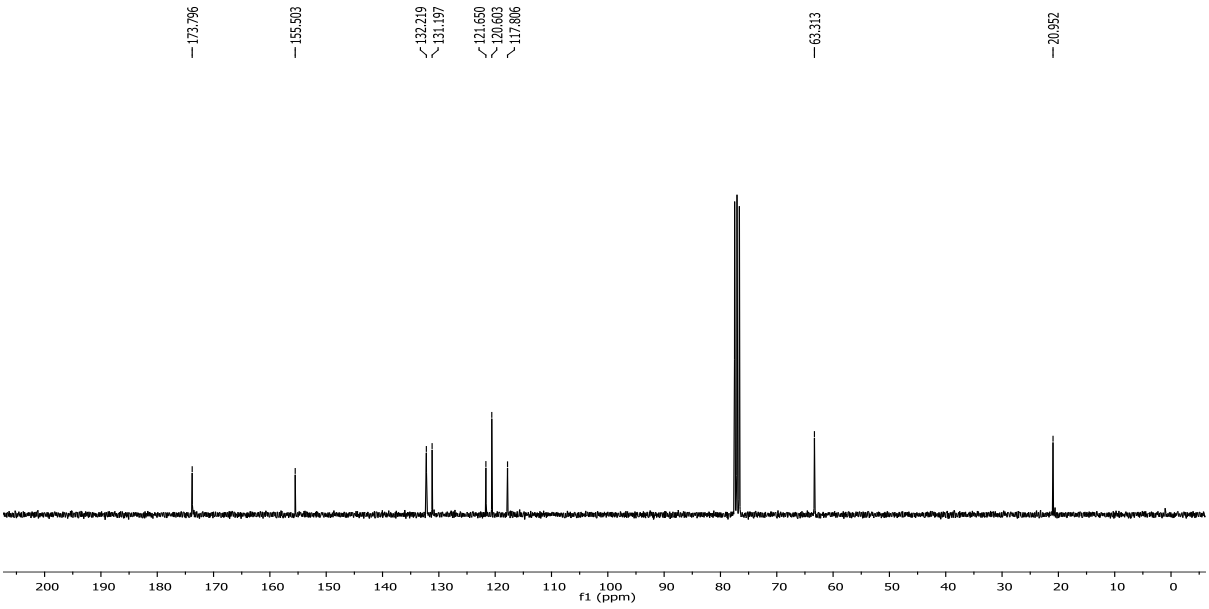


Figure 7.1c: ^{13}C NMR plot showing 2-hydroxybenzyl acetate

Analysis of ^{1}H and ^{13}C NMR of 2-hydroxybenzyl acetate

^{1}H analysis: The singlet peak at 2.13 ppm with integration for three protons was assigned to the CH_3 group of ester. CH_2 protons adjacent to benzene ring showed a singlet peak at 5.14 ppm. Mutiplets at 6.94 ppm (2H) and 7.30 ppm (2H) indicated presence of aromatic protons in the molecule and the pattern indicates o-substituted benzene ring. Presence of phenolic hydroxyl was indicated by a broad singlet at 7.82 ppm, which disappeared on adding 1-2 drops of D_2O to the NMR tube.

^{13}C analysis: The peak at 20.95 ppm was assigned to the CH_3 of ester carbon. Peak at 63.31 ppm indicated presence of CH_2 group between ester and benzene ring. Peak at 173.80 ppm was assigned to the carbonyl carbon of the ester. The low intensity peaks at 155.50 ppm and 121.65 indicated presence of quaternary carbon atoms in the benzene ring. High intensity peaks at 117.81 ppm, 120.60 ppm, 131.20 ppm and 132.22ppm showed existence of unsubstituted aromatic carbon atoms.

Yield 74.2 %, colorless liquid, TLC R_f = 0.5 (EtOAc: n-Hexane, 6:4);^{1}H NMR (300 MHz, $CDCl_3$) δ 2.13 (CH_3, s, 3H), 5.14 (CH_2, s, 2H), 6.94 (Ar, m, 2H), 7.30 (Ar, m, 2H), 7.82 (OH, s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (CH_3)20.95, (CH_2)63.31, 117.81, 120.60, 121.65, 131.20, 132.22, 155.50, 173.80; FT-IR Wavenumber [cm^{-1}] 3401, 2924, 1793, 1706, 1593, 1503, 1455, 1377, 1256, 1095, 1039, 928, 822, 754, 609.396, 528.4, 434.869.

3-hydroxybenzyl acetate (3-HBA)

IR Wavenumber [cm^{-1}] 3364.21, 2878.24, 1717.3, 1594.84, 1458.89, 1278.57, 1159.01, 1034.62, 921.807, 865.882, 785.85, 750.174, 695.212.

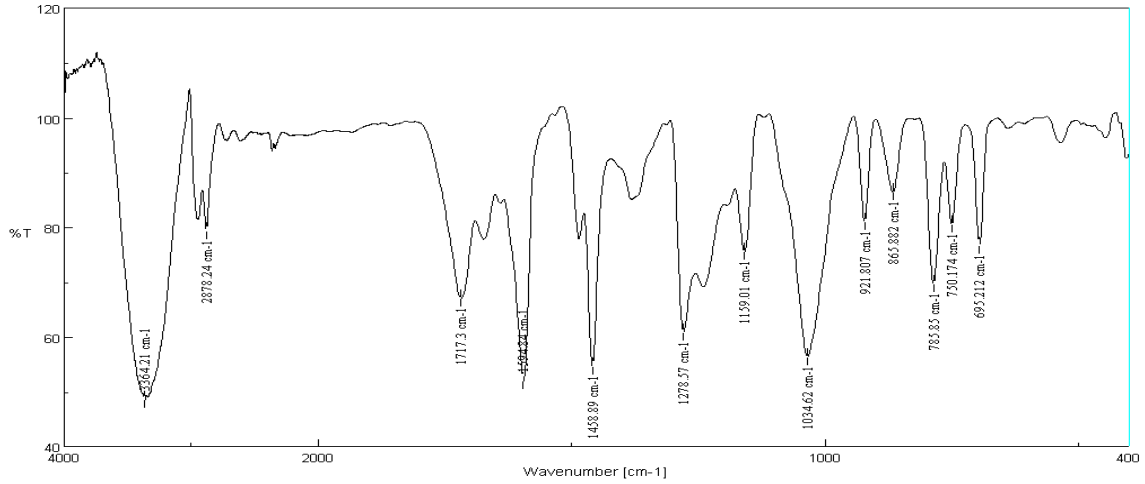


Figure 7.2a: IR plot showing 3-hydroxybenzyl acetate

^{1}H NMR (300 MHz, $CDCl_3$) δ 2.14 (s, 3H), 5.09 (s, 2H), 6.04 (s, 1H), 6.84 (dd, *J* = 12.2, 4.1 Hz, 2H), 6.92 (d, *J* = 7.6 Hz, 1H), 7.25 (dd, *J* = 12.7, 4.9 Hz, 1H).

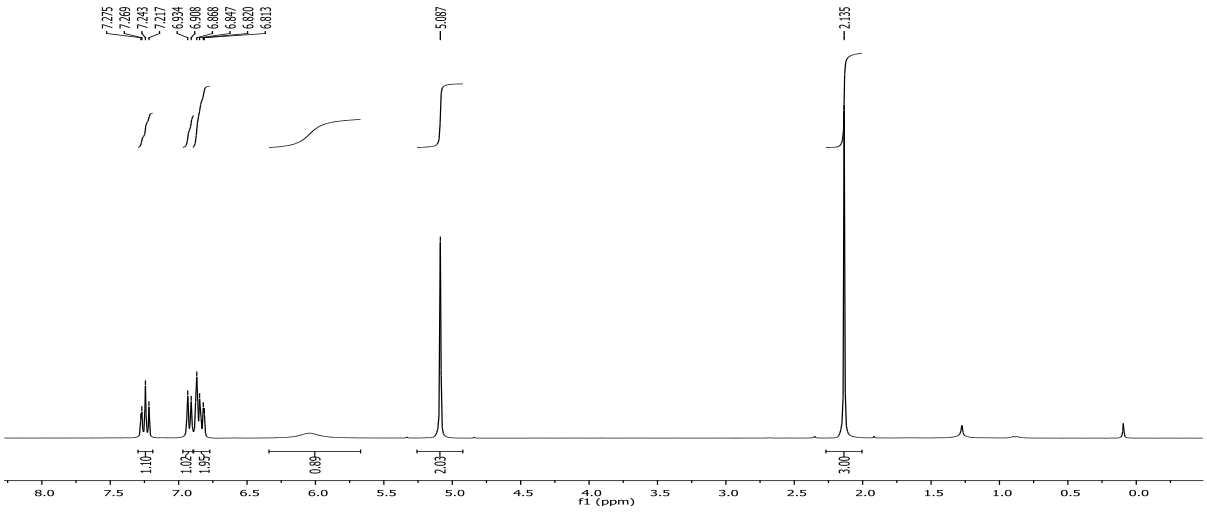


Figure 7.2b: ^{1}H NMR plot showing 3-hydroxybenzyl acetate

^{13}C NMR (75 MHz, $CDCl_3$) δ 21.09, 66.30, 115.13, 115.38, 120.34, 129.90, 137.42, 156.01, 171.70.

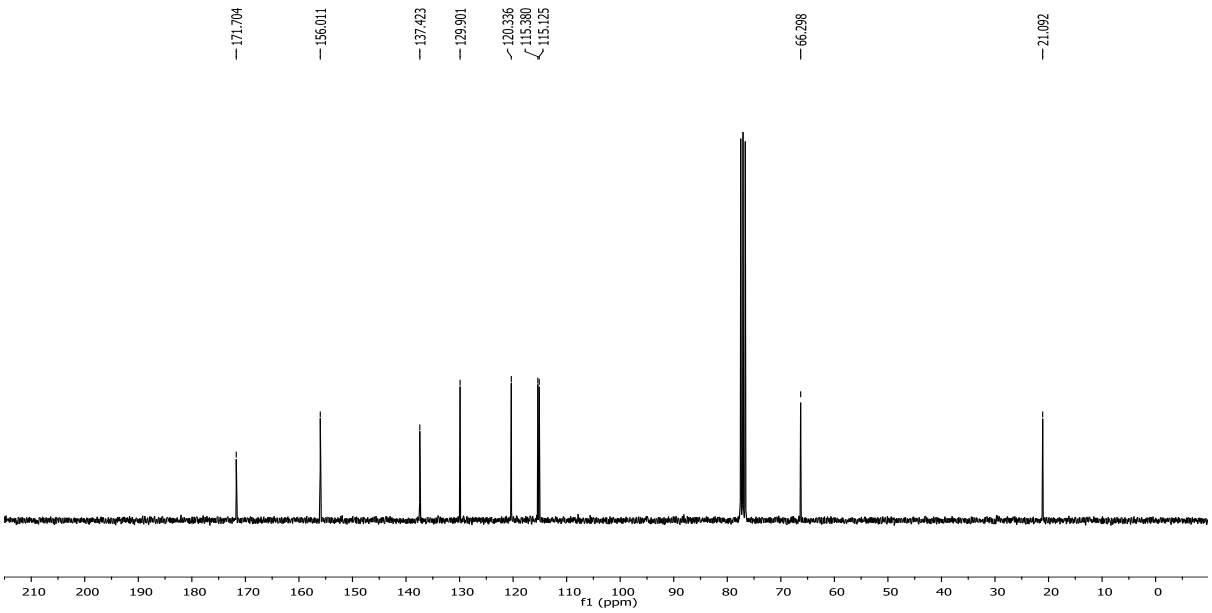


Figure 7.2c: ^{13}C NMR plot showing 3-hydroxybenzyl acetate

Analysis of ^{1}H and ^{13}C NMR of 3-hydroxybenzyl acetate

^{1}H analysis: The singlet peak at 2.14 ppm with integration for three protons was assigned to the CH_3 group of ester. CH_2 protons adjacent to benzene ring showed a singlet peak at 5.09 ppm. Presence of phenolic hydroxyl was indicated by a broad singlet at 6.04 ppm. Multiplet, doublet and triplet at 6.84 ppm (2H), 6.92 ppm (1H, J = 7.6Hz) and 7.25 ppm (1H, J = 12.7 Hz), respectively indicated presence of aromatic protons in the molecule.

^{13}C analysis: The peak at 21.09 ppm was assigned to the CH_3 of ester carbon. Peak at 66.30 ppm indicated presence of CH_2 between ester and benzene ring. Peak at 171.70 ppm was assigned to the carbonyl carbon of the ester. The low intensity peaks at 156.01 ppm and 137.42

indicated presence of quaternary carbon atoms in the benzene ring. High intensity peaks at 115.13 ppm, 115.38 ppm, 120.34 ppm and 129.90 ppm showed existence of unsubstituted aromatic carbon atoms.

Yield 78.7 %, colorless liquid, TLC R_f = 0.5 (EtOAc: n-Hexane, 5:5);^{1}H NMR (300 MHz, $CDCl_3$) δ 2.14 (CH_3, s, 3H), 5.09 (CH_2, s, 2H), 6.04 (OH, s, 1H), 6.84 (m, 2H), 6.92 (d, *J* = 7.6 Hz, 1H), 7.25 (dd, *J* = 12.7, 4.9 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 21.09, 66.30, 115.13, 115.38, 120.34, 129.90, 137.42, 156.01, 171.70; FT-IR Wavenumber [cm^{-1}] 3364, 2878, 1717, 1594, 1458, 1278, 1159, 1034, 921, 865, 785, 750, 695.

4-hydroxybenzyl acetate (4-HBA)

IR Wavenumber [cm^{-1}] 3373.85, 3026.73, 2957.3, 1710.55, 1615.09, 1597.73, 1518.67, 1450.21, 1383.68, 1362.46, 1235.18, 1171.54, 1106.94, 1025.94, 959.412, 849.49, 827.312, 777.172, 609.396.

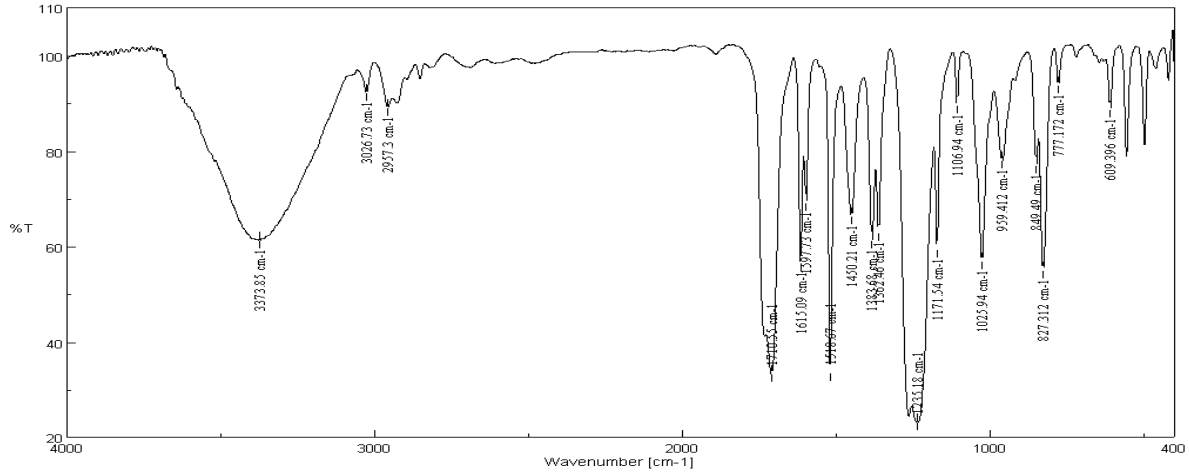


Figure 7.3a: IR plot showing 4-hydroxybenzyl acetate

^{1}H NMR (300 MHz, $CDCl_3$) δ 2.11 (s, 3H), 5.05 (s, 2H), 6.86 (t, *J* = 7.9 Hz, 2H), 7.37 – 7.17 (m, 2H).

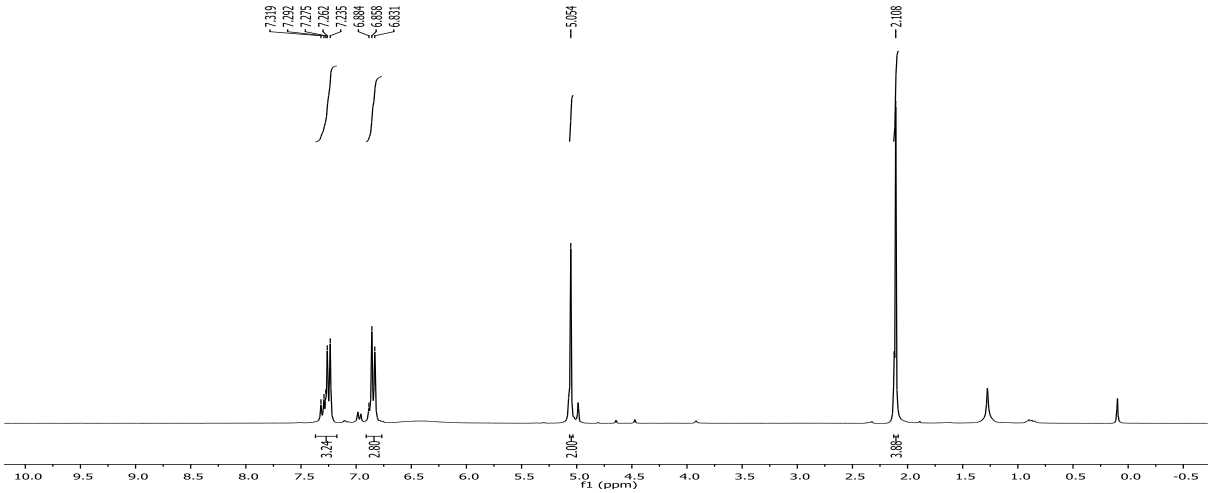


Figure 7.3b: ^{1}H NMR plot showing 4-hydroxybenzyl acetate

^{13}C NMR (75 MHz, $CDCl_3$) δ 21.18, 66.53, 115.50, 127.63, 130.41, 156.12, 171.92.

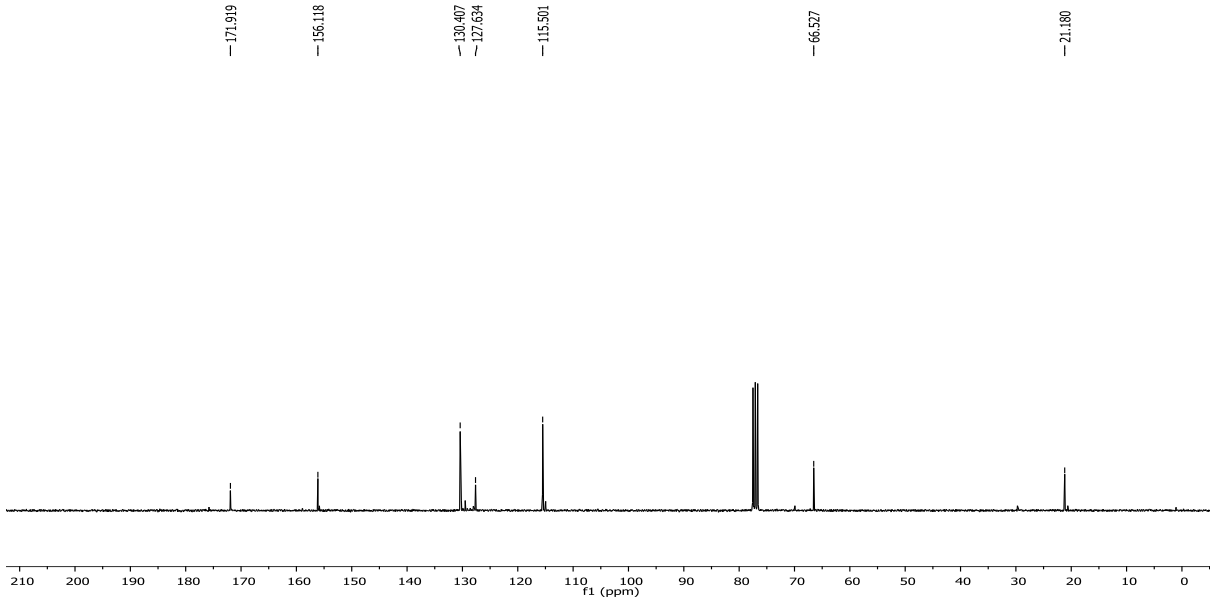


Figure 7.3c: ^{13}C NMR plot showing 4-hydroxybenzyl acetate

Analysis of ^{1}H and ^{13}C NMR of 4-hydroxybenzyl acetate

^{1}H analysis: Singlet peak at 2.11 ppm and 5.05 ppm indicated presence of CH_3 group of ester and CH_2 group, respectively. Doublets at 6.86 ppm (2H, J = 8.1 Hz) and 7.17 ppm (2H, J = 8.1 Hz) indicated presence of aromatic protons in the molecule and the pattern indicates p-

substituted benzene ring. Presence of phenolic hydroxyl was indicated by a broad singlet at 6.35 ppm.

^{13}C analysis: The peak at 21.18 ppm was assigned to the CH_3 of ester carbon. Peak at 66.53 ppm indicated the presence of CH_2 between ester and benzene ring. Peak at 171.92 ppm was assigned to the carbonyl carbon of the ester. The low intensity peaks at 156.12 ppm and 127.63 ppm indicated presence of quaternary carbon atoms in the benzene ring. High intensity peaks at 115.50 ppm and 130.4 ppm showed existence of unsubstituted aromatic carbon atoms.

Yield 70 %, colorless liquid, TLC R_f = 0.4 (EtOAc: n-Hexane, 5:5); ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.11 (CH_3, s, 3H), 5.05 (CH_2, s, 2H), 6.86 (m, 2H), 7.37 – 7.17 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 21.18, 66.53, 115.50, 127.63, 130.41, 156.12, 171.92; IR Wavenumber [cm-1] ;3401.82, 2924.52, 1793.47, 1706.69, 1593.88, 1503.24, 1455.03, 1377.89, 1256.4, 1095.37, 1039.44, 928.557, 822.491, 754.031, 609.396, 528.4, 434.869.

2-phenyl-4- (D-arabino-4'-acetoxy-1', 2', 3'-trihydroxybutyl) -2H- 1, 2, 3-triazole (GTRIA)

IR wavenumber [cm^{-1}] 3392.17, 2955.38, 2916.81, 2848.35, 1734.66, 1519.63, 1472.38, 1462.74, 1365.35, 1231.33, 1024.98, 728.961, 719.318, 420.406.

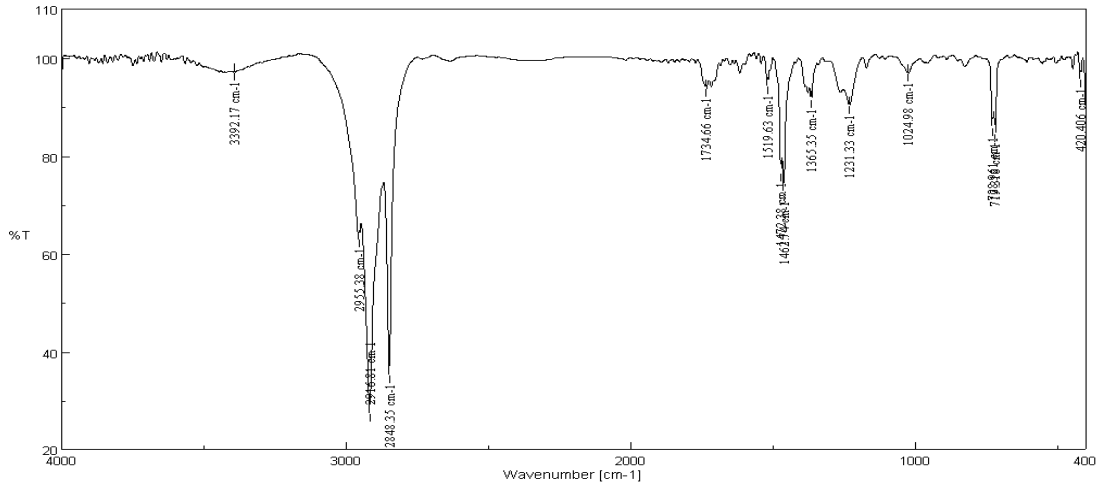


Figure 7.4a: IR plot showing triazole acetate

^{1}H NMR (600 MHz, DMSO) δ 2.03 (s, 3H), 3.56 – 3.53 (m, 1H), 3.85 – 3.81 (m, 1H), 7.97 (s, 1H), 5.37 (d, *J* = 7.0 Hz, 1H), 4.85 (d, *J* = 7.9 Hz, 1H), 7.40 (t, *J* = 7.4 Hz, 1H), 7.57 – 7.55 (m, 2H), 7.99 (d, *J* = 7.7 Hz, 2H), 4.27 (dd, *J* = 11.3, 2.5 Hz, 1H), 4.00 (dd, *J* = 11.3, 6.5 Hz, 1H), 5.11 (dd, *J* = 7.1, 2.0 Hz, 1H), 5.09 (d, *J* = 6.2 Hz, 1H).

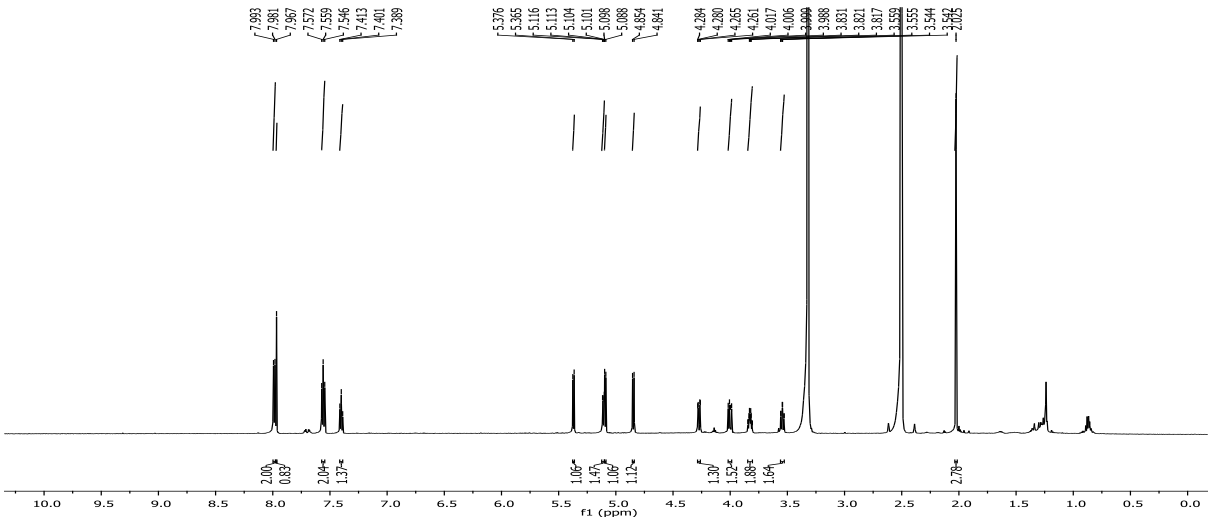


Figure 7.4b:^{1}H NMR plot showing triazole acetate

^{13}C NMR (151 MHz, DMSO) δ 21.33, 65.65, 66.97, 68.50, 74.30, 118.56, 127.80, 130.14, 135.80, 139.78, 153.48, 171.03.

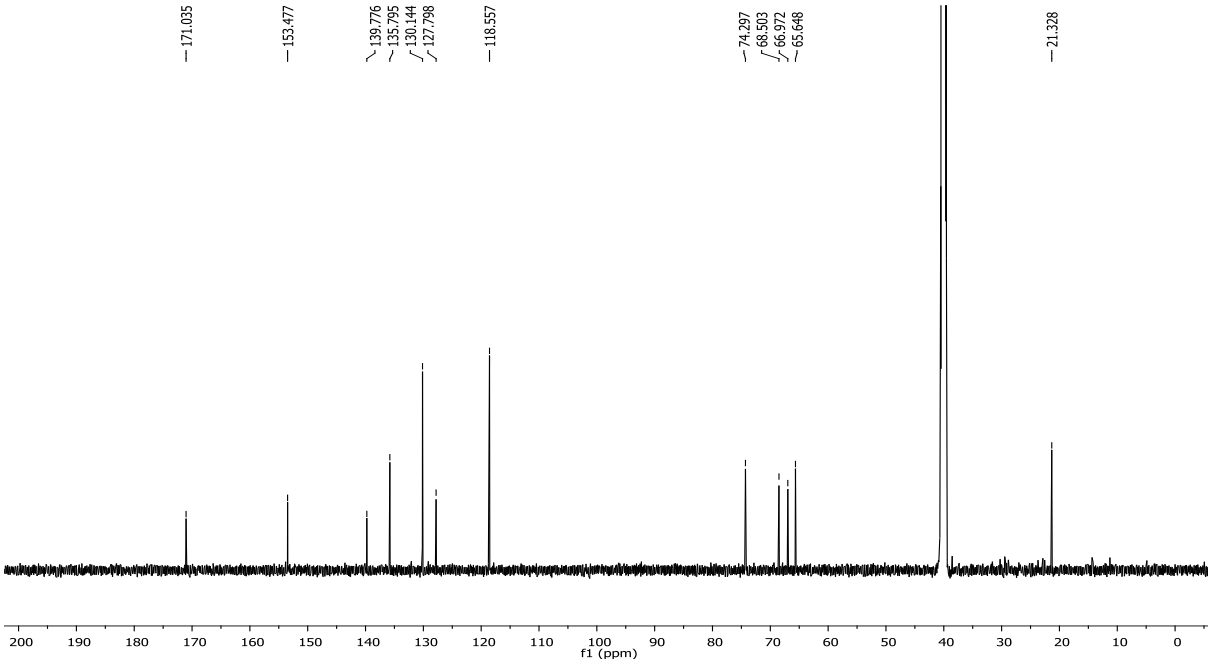


Figure 7.4c:^{13}C NMR plot showing triazole acetate

Analysis of ^{1}H and ^{13}C NMR of Triazole acetate

^{1}H analysis: Singlet peak at 2.02 ppm indicated presence of CH_3 group of ester. Peaks from 3.54 to 4.01ppm were assigned to hydroxyl groups in the molecule. These peaks disappeared when treated with D_2O. Peaks from 4.28 to 5.37 ppm indicated presence of one CH_2 and three CH functionalities in the molecule. Presence of aromatic protons in the molecule was indicated by peaks between 7.38 to 8.00 ppm. Hydrogen attached directly to triazole carbon is indicated by a singlet peak at 7.96 ppm.

^{13}C analysis: The peak at 21.32 ppm was assigned to the CH_3 of ester carbon. Peaks at 65.64, 66.97, 68.50 and 74.29 ppm indicated presence of one CH_2 and three CH functionalities. Peaks at 118.55, 127.79, 130.14, 135.79, 139.77 and 153.47 ppm suggested presence of aromatic ring in the molecule. Presence of carbonyl carbon was indicated by peak at 171.03 ppm.

Yield 16.66 %, White solid, TLC R_f = 0.3 (EtOAc); ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.03 (s, 3H), 3.56 – 3.53 (m, 1H), 3.85 – 3.81 (m, 1H), 7.97 (s, 1H), 5.37 (d, *J* = 7.0 Hz, 1H),

4.85 (d, *J* = 7.9 Hz, 1H), 7.40 (t, *J* = 7.4 Hz, 1H), 7.57 – 7.55 (m, 2H), 7.99 (d, *J* = 7.7 Hz, 2H), 4.27 (dd, *J* = 11.3, 2.5 Hz, 1H), 4.00 (dd, *J* = 11.3, 6.5 Hz, 1H), 5.11 (dd, *J* = 7.1, 2.0 Hz, 1H), 5.09 (d, *J* = 6.2 Hz, 1H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 21.33, 65.65, 66.97, 68.50, 74.30, 118.56, 127.80, 130.14, 135.80, 139.78, 153.48, 171.03; IR Wavenumber [cm^{-1}] 3392.17, 2955.38, 2916.81, 2848.35, 1734.66, 1519.63, 1472.38, 1462.74, 1365.35, 1231.33, 1024.98, 728.961, 719.318, 420.406.

7.4 Discussion

Degradation of fat is an important property of lipases. From the literature it is evident that the lipase produced from *Lactobacillus* sp. had the property to degrade meat in 72 hrs [263]. Similarly the proteolytic activity of lipase towards meat proteins in sausage system was studied and *L. plantarum* showed degradation of both sarcoplasmic and myofibrillar proteins in 96 hrs [264]. Complete degradation in the present study was observed at 96 hrs for *L. brevis* and 72 hrs for *L. plantarum* as shown in the Table 7.1 and co-culture of recombinant lipase showed complete degradation in 48 hrs with strong smell and froth. This might be due to autooxidation of fattyacids into short chain aldehydes or ketones leading to objectionable smell and frothing.

Figures 7.1 (a-c) to 7.4 (a-c) represented the synthesis of 2, 3, 4-hydroxybenzyl acetate and Triazole acetate (short chain fatty acid esters) which were confirmed by IR and NMR data analysis. All these esters are used as flavoring agents which can be applied in food industry. 4-hydroxybenzyl acetate can also be used in cosmetics as anti-tanning agent.

7.5 Conclusion

From the above results it was evident that the co-cultured lipase is more efficient than the individual cultures in removing fat in medical field and also degrading lipid containing waste water preventing water pollution. From the transesterification reactions it was evident that lipase

from *Lactobacillus plantarum* was novel in synthesizing different short chain fatty acids esters which are used as flavoring agents in food industry.

Summary and Conclusions

8.1 Summary

Lipases constitute one of the most industrially important enzymes which are widely used in detergents, beverages, dairy, cosmetics, biofuel industry, biopolymer synthesis, biodiesel production, treatment of fat-containing waste effluents, enantiopure synthesis of pharmaceuticals and nutraceutical agents. They may be of animal, plant or microbial origin. Because of the huge variations in applications, the availability of lipases with specific characters is still a limiting factor. Hence, the present study aims to explore different microorganisms that produce lipase and optimization of the culture parameters for maximum lipase production. This was followed by heterologous expression, purification, immobilization and kinetic study of the enzyme. Finally the lipase was used to synthesizeshort chain fatty acid esters which find use as flavoring agent in food industry.

The main objective of the present work is to produce extra-cellular lipase. The objective of the present work can thus be broadly divided into the following aspects:

- Selection and optimization of physical parameters for lipase production from Lactic acid bacteria by classical and statistical methods.
- Strain improvement by mutagenesis and comparative study of physical-chemical parameters of parent and mutant strains.
- Heterologous expression and purification of lipase.
- Immobilization and kinetic study of expressed and immobilized lipase.
- Lipase mediated ester synthesis and their industrial applications.

i) Selection and optimization of physical parameters for lipase production from Lactic acid bacteria by classical and statistical methods

The major objective of the present work was to optimize the environmental parameters for lipase production by single and co-cultures of lactic acid bacteria. In course of the optimization studies it was observed that among nonedible oils, olive oil was found to be the best suitable substrate for maximum lipase production. The optimum operating conditions using statistical tools were close to the single variable optimization results. The lipase activity with classical method of optimization was 32 U/ml from co-culture of *L. lactis* and *L. brevis*, 35 U/ml for *L. lactis* and *L. plantarum* and 37 U/ml for *L. brevis* and *L. plantarum* whereas with the above statistical approach the activity was found to be 33.5 U/ml from co-culture of *L. lactis* + *L. brevis* and 36 U/ml from *L. lactis* + *L. plantarum* and 38.5 U/ml for *L. brevis* and *L. plantarum* which showed 2 fold increase in the lipase production. The above results prove that the statistical approach showed better results to maximize the lipase production.

ii) Strain improvement by mutagenesis and comparative study of physical-chemical parameters of parent and mutant strains

In order to improve the productivity of the parent strain it was subjected to mutagenic studies with mutagens like UV, heat treatment, EtBr. increased activity of enzyme was not observed with heat treatment and EtBr mutagenesis whereas with 120 min incubation of UV, there is increase in the lipase activity of the three mutant co-culture combinations showing *L. lactis* + *L. brevis as* 34 U/ml, *L. lactis* + *L. plantarum as* 35 U/ml and *L. brevis* + *L. plantarum as* 39 U/ml respectively when compared to the individual normal controls. 2 U/ml increase in activity of lipase was observed.

Then a comparative study of the biochemical parameters of both parent and mutant strains were carried out. Thereafter it was found that the mutants were found to be a hyper producer of protease and amylase in addition to lipase. Thus, from this study it was evident that the UV mutagenesis had some effects not on the morphology but on the biochemical characters of the organism.

iii) Heterologous expression and purification of lipase

Lipase genes were amplified and cloned into vector pMALc5X and expressed in expressed in *E. coli* BL21 (DE3) pLysS. The specific activity of lipase before expression was 12.5 U/mg (enzyme activity was 35 U/ml) for *L. brevis* and 13.2 U/mg (enzyme activity 37 U/ml) for *L. plantarum*. The expressed lipase of *L. brevis* showed 3 fold increase in specific activity of 36.42 U/mg (enzyme activity 102 U/ml) after expression and similarly the expressed lipase of *L. plantarum* showed 3.3 fold increase in specific activity of 42.8 U/mg (enzyme activity 110 U/ml) after expression.Then the molecular weights of the strains were obtained by SDS-PAGE. *L.brevis* showed a molecular weight of 27 KDa and the lipase enzyme from *L. plantarum* showed a molecular weight of 66 KDa.

iv) Immobilization and kinetic study of expressed and immobilized lipase

In order to know the characteristics of the enzyme, it is very important to study its kinetics. From the above studies it was found that the sodium alginate gel was suitable method of immobilization of lipase enzyme. The expressed and immobilized lipase enzyme was active at pH 7 for *L. brevis* and pH 6 and pH 6.5 for *L. plantarum.* The optimum activity of the expressed and immobilized enzyme was found to be 37 °C and 40 °C for *L. brevis* and 40 °C and 45 °C for *L. plantarum*. The K_m and V_{max} values at the optimum temperatures for both wild and mutant strains were also found using Lineweaver-Burk plot.

v) Lipase mediated ester synthesis and their industrial applications

Industrial applications of the immobilized lipase from *L. brevis* and *L. plantarum* and the esterification reaction using these lipases were studied. In fat degradation study it was found that complete degradation of fat was observed at 96 hrs for *L. brevis* and 72 hrs for *L. plantarum* and lipase from co-culture showed complete degradation in 48 hrs. From NMR and IR data, the peaks that were obtained represented the synthesis of 2, 3, 4-hydroxybenzyl acetates and Triazole esters which can be used as flavor agents in food industries.

8.2 Conclusion

In conclusion the following observations may be made about the results obtained in this study.

1. Firstly, the optimization of physical parameters relating to lipase production was studied using classical OFAT method and statistical tools like RSM, ANN, GA, Taguchi and SVM methods. From the study it was evident that statistical approach was more beneficial in the production of lipase from both single and co-cultures of different lactic acid bacteria when compared to the classical OFAT optimization to enhance the lipase production by 2 fold times.
2. Further, the improvement of parent strain by mutagenesis had been carried out by mutagens like UV, heat treatment and EtBr to obtain a stable hyper-producing mutant where UV irradiated mutant showed an increase in lipase activity of 2 U/ml. Though not much improvement in lipase activity was observed when compared to parent strain the mutant strains possessed novel properties of large-scale production of both protease and lipase enzymes.
3. Thereafter, heterologous expression was done which was successful and showed a 3 fold increase in lipase activity when compared to the native form. Recombinant DNA technology was found to be best method for strain improvement.
4. Immobilization of lipase by entrapment method using sodium alginate was found to be suitable, cheap and the molecular weights of the proteins were determined by SDS-PAGE. Then, the enzyme kinetics of the strains was studied.
5. Lipase from co-culture was found to be more efficient than the individual cultures. This lipase was used in removing fat useful in medical field and also degrading lipid

containing waste water that prevents water pollution. Transesterification by lipase from *Lactobacillus plantarum* was novel in synthesizing different short chain fatty acid esters which are used as flavoring agents in food industry.

8.3 Major contributions of the work

The following are the major contributions from the above work

- There are several reports on production of lipase from single cultures of bacteria and fungi. Similarly production of lipase from co-culture of different fungi was also reported. But to the best of our knowledge this was the first report on optimization of culture conditions influencing the production of lipase from co-cultures of *Lactococcuslactis, Lactobacillus plantarum* and *Lactobacillus brevis.*
- Multi factorial optimization was done by statistical tools like RSM, ANN, GA, Taguchi and SVM methods where the optimization data paved the path for large scale industrial production of lipase enzyme and showed 2 fold increased activity.
- Heterologous expression of lipase further showed 3 fold increase in activity compared to the native form.
- The use of co-cultured lipase in the degradation of fat makes it useful in medical field and also degrading lipid containing waste water thus preventing water pollution.
- Short chain fatty acid ester extraction from plants is a highly expensive process. The cost of such a process was minimized in this study by the production of cost effective short chain fatty acid esters using our microbial lipase. These esters find use as flavoring agents in food industry and in cosmetics.

8.4 Future scope of work

The future possibilities arising from the results of the present work are as given below:

- Microbial consortia in place of co-culture may be used to study the lipase production.
- The potent UV irradiated mutant obtained in the study may be further harnessed for industrial applications due to its capability to produce multiple enzymes.
- Other immobilization techniques can further be studied to check the functional efficacy of the lipase for synthesizing industrially important compounds.
- Synthesis of lipase mediated long chain fatty acid esters, amides and polymer degradation can be further studied.

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BIOGRAPHY OF SUPERVISOR

Dr. Jayati Ray Dutta, Assistant Professor of Biological Sciences, has been with Birla Institute of Technology and Science, Pilani, Hyderabad Campus, India since 2008.She obtained her Master's degree from Calcutta University and Ph. D from Indian Institute of Technology (IIT), Kharagpur, India in 2004. She also served as DST FAST TRACK Young Scientist in IIT, Kharagpur, India till July, 2008.

Dr. Ray Dutta's research focus is on environmental biotechnology, microbial enzymology and their biotechnological applications. Beginning her professional career in 2000, Dr. Ray Dutta has little more than 14 years of academic and industry experience in the field of Microbial Biotechnology. She has published over 26 research papers with good citations in reputed international journals and conferences. She has been member of academic societies and associations including European Federation of Biotechnology and Biotech Research Society of India. She has been editorial board member and reviewer for many International Journals. Currently, her group is engaged in the development of industrially important enzymes like lipases and proteases which are natural biocatalysts from different microbial sources for commercial purposes. She has successfully completed research projects and ongoing projects sponsored by DST, DBT and UGC, India.

Administrative experience

1. Convener, Departmental Research Committee - 2011 - till date
2. Member of Departmental Committee on Academics
3. Member of Central Research Facility - 2008 - till date
4. In-Charge, Biology Laboratory, Biological Sciences (Since, 2011)

Dissertation Guidance:

1. PhD students: 1 to start and 1 completed
2. M.E. student: 3
3. B.Tech student: 3

SOP (Study oriented project), LOP (Laboratory oriented project):

1. SOP students: 5 completed
2. LOP students: 3 completed

Reviewer for journals:

Biotechnology and Bioprocess Engineering Journa

Journal of Food, Science and Technology

Process Biochemistry

Bioresource Technology

Waste & Biomass Valorization Journal

Journal Publications:

1. **Jayati Ray Dutta**, P. K. Dutta and R. Banerjee, Optimization of culture parameters for extra-cellular protease production from a newly isolated *Pseudomonas sp.* using response surface and artificial neural network models. *Process Biochemistry*, 39, 12 (2004), pp. 2193-2198.
2. **Jayati Ray Dutta**, P. K. Dutta and R. Banerjee, Modeling and optimization of protease production by a newly isolated *Pseudomonas* sp. using a genetic algorithm. *Process Biochemistry*, 40, 2, 2005, pp. 879-884.

3. **Jayati Ray Dutta**, P. K. Dutta and R. Banerjee: Kinetic study of a low molecular weight protease from a newly isolated *Pseudomonas* sp. using artificial neural network. *Indian Journal of Biotechnology*, 4, 1, 2005 pp. 127-133.
4. **Jayati Ray Dutta** and R. Banerjee: Isolation and characterization of a newly isolated *Pseudomonas* mutant for protease production. *Brazilian Archives of Biology and Technology*, 49, 1, 2006, pp. 37-47.
5. J. Purohit, **Jayati Ray Dutta**, and R. Banerjee: Strain improvement for tannase production from co-culture of *Aspergillus foetidus* and *Rhizopus oryzae. Bioresource Technology*, 97, 6, 2006, pp. 795-801.
6. S. K. Hota, **Jayati Ray Dutta**, and R. Banerjee: Immobilization of tannase from *Rhizopus oryzae* and its efficiency to convert different tannin rich agro-residues to gallic acid. *Indian Journal of Biotechnology*, 6, 2, 2007, pp. 200-204.
7. **Jayati Ray Dutta** and U. Sita Ramyasri, Optimization of L-arabinose isomerase production from *Lactococcus lactis lactis*: bioconversion of D-galactose to D-tagatose using the enzyme, *International Journal of Biosciences*, 2, 10, 2012, pp. 48-57.
8. Sita Ramyasree Uppada, Amit Kumar Gupta, **Jayati Ray Dutta**, Statistical optimization of culture parameters for lipase production from *Lactococcus lactis* and its application in detergent industry, *International Journal of ChemTech Research*, 4, 4, 2012, pp. 1509-1517.
9. Sita Ramyasree Uppada and **Jayati Ray Dutta**, The effect of process parameters in enhancement of lipase production by co-culture of lactic acid bacteria and their mutagenesis study, *Biocatalysis and Agricultural Biotechnology*, 2, 2013, pp. 393-398.

10. Sita Ramyasree Uppada, Aditya Balu, Amit Kumar Gupta and **Jayati Ray Dutta**, Modeling lipase production from co-cultures of lactic acid bacteria using neural networks and support vector machine with genetic algorithm optimization, *International Journal of Emerging Technologies in Computational and Applied Sciences*, 9, 2014, pp. 38-43.
11. Sita Ramyasree Uppada, Aditya Balu, Amit Kumar Gupta and **Jayati Ray Dutta**, Hybrid neural network based optimization of process parameters and application of Taguchi method for lipase production from co-culture of *Lactobacillus brevis* and *Lactobacillus plantarum*, *International Journal of PharmaTech Research*, 6, 2014, pp.2074-2082.
12. Sita Ramyasree Uppada and **Jayati Ray Dutta**, Immobilization and kinetic study of lipase enzymes from *Lactobacillus plantarum* and *Lactobacillus brevis*, *International Journal of ChemTech Research*, 8, 2015, pp. 680-685.

Articles in Magazine:

13. **Jayati Ray Dutta**: The Evolution of Biotechnology and its Impact on Social Welfare *National Science Day*, Nehru Museum Publication, IIT Kharagpur, 2003.
14. **Jayati Ray Dutta**: Response surface and artificial neural network based optimization of culture parameters for protease production, *National Science Day*, Nehru Museum Publication, IIT Kharagpur, 2004.

Conference Papers/Symposium Attended:

1. **Jayati Ray Dutta**, P. K. Dutta and R. Banerjee: Modeling and optimization of protease production from a newly isolated *Pseudomonas sp.* using genetic algorithm – In Systems Conference, 2003, IIT Kharagpur, Dec 17-19, pp. 353-357.

2. Ashish Sachan, **Jayati Ray Dutta**, Nirupama Mallick and R. Banerjee: Production of value-added product from tannery waste – In "Emerging Technology for Sustainable Environment in Chemical and Allied Industries" 2004, NIT Rourkela, Oct 2-3.
3. **Jayati Ray Dutta**, Manisha Mishra and R. Banerjee: Microbial production of tagatose: a low calorie sweetener- In National Conference on Path to Health Biotechnology Revolution in India, 2005, Anna University, Chennai, November 24-26.
4. **Jayati Ray Dutta**, P. K. Dutta and R. Banerjee: Kinetics of low molecular weight proteases from newly isolated *Pseudomonas sp*. and its mutant using artificial neural network – In Asia Pacific Biochemical Engineering Conference on "Biochemical Engineering from Genomics to Human Well-being" 2005, Korea, May 15-19.
5. **Jayati Ray Dutta**, P.K. Dutta and R. Banerjee: Optimization of protease production from a new isolate *Pseudomonas sp.* by genetic algorithm- In European Conference on Mathematical and Theoretical Biology, 2005, Dresden, Germany, July 18-22.
6. U. Sita Ramyasri and **Jayati Ray Dutta**: Enzymatic transesterification of non-edible oils for the production of biofuels – In contemporary trends in Biological and Pharmaceutical Research (CTBPR-2011) Conference, 2011, BITS-Pilani, Rajasthan, March 12-13.
7. U. Sita Ramyasri and **Jayati Ray Dutta**: Statistical optimization of culture conditions by Response surface methodology, Artificial neural network and Genetic algorithm for synthesis of lipase from *Lactococcus lactis* – In World Congress Biotechnology, Bright International Conference, 2012, Leonia International Convention Centre, Hyderabad, India, May 4-6.
8. U. Sita Ramyasri, Harshitha and **Jayati Ray Dutta**: Optimization of culture conditions for the production of lipase by co-culture of *Lactobacillus brevis and Lactococcus lactis* -

In International Conference on Advances in Chemical Engineering (ICACE, 2013), NIT, Raipur, Chhattisgarh, India, April 5-6.

9. U. Sita Ramyasri and **Jayati Ray Dutta**: Lipase mediated ester synthesis from different lactic acid bacteria through coculture technique - In International Conference on Biodiversity, Bioresources and Biotechnology, 2014, Mysore, India, Jan 30-31.
10. U. Sita Ramyasri and **Jayati Ray Dutta**: Application of expressed lipase from *Lactobacillus plantarum* - In International Symposium on "Proteomics: Present and Future" 2014, CCMB, Hyderabad, India, Nov 22-24.
11. U. Sita Ramyasri and **Jayati Ray Dutta**: Microbial Lipase mediated ester synthesis for its industrial application-2nd International Conference and Exhibition on Biotechnology, BRIGHTICE, 2015, Leonia, Hyderabad, India, Aug 3-4.

Workshop Attended:

12. Workshop on Innovations in Waste, Water and Energy Technologies for Rural Development, 2015, BITS-Pilani, Hyderabad, July 13-14.

Awards and Distinctions:

1) Recipient of National Scholarship for post-graduation studies from Calcutta University (1995).
2) Best presentation award in a session, NSC, conference 2003 in IIT Kharagpur.
3) Recipient of Young Scientist Fast Track Award from Department of Science &Technology, Govt. of India (2006-2009).
4) Biographical profile published in the Marquis Who's Who in the World (2009)
5) Best Poster Presentation Award in the International Conference on advances in Chemical Engineering (ICACE - 2013), NIT, Raipur, April, 5-6, 2013.

6) Special Jury Award for oral presentation on “Lipase mediated ester synthesis from different lactic acid bacteria through co-culture technique” in the International Conference on Biodiversity, Bioresources and Biotechnology, Mysore, India, Jan 30-31, 2014.

BIOGRAPHY OF Ms. U. SITA RAMYASRI

Ms. U. Sita Ramyasri obtained her Master's degree (M.Sc) in Microbiology from Andhra University, Visakhapatnam, India in 2008 and started her research as a research fellow in the Department of Biological Sciences at BITS Pilani, Hyderabad Campus, India in 2011. She is well versed in various Microbiology and Recombinant DNA technology methods. She has good number of publications and awards to her credit and has presented her work in several national and international conferences. Currently, her research interests focus on the development of genetically distinct lipases from different Lactic acid bacteria and lipase mediated ester synthesis by co-culture technology.

Journal Publications:

1. **Sita Ramyasree Uppada** and Jayati Ray Dutta, Immobilization and kinetic study of lipase enzymes from *Lactobacillus plantarum* and *Lactobacillus brevis*, *International Journal of ChemTech Research*, 8, 2015, pp. 680-685.
2. **Sita Ramyasree Uppada**, Aditya Balu, Amit Kumar Gupta and Jayati Ray Dutta, Hybrid neural network based optimization of process parameters and application of Taguchi method for lipase production from co-culture of *Lactobacillus brevis* and *Lactobacillus plantarum*, *International Journal of PharmaTech Research*, 6, 2014, pp.2074-2082.
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4. **Sita Ramyasree Uppada** and Jayati Ray Dutta, The effect of process parameters in enhancement of lipase production by co-culture of lactic acid bacteria and their mutagenesis study, *Biocatalysis and Agricultural Biotechnology*, 2, 2013, pp. 393-398.
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Conference Papers/Symposia:

1. **U. Sita Ramyasri** and Jayati Ray Dutta: Microbial Lipase mediated ester synthesis for its industrial application-2nd International Conference and Exhibition on Biotechnology, 2015, Leonia, Hyderabad, India, Aug 3-4.
2. **U. Sita Ramyasri** and Jayati Ray Dutta: Application of expressed lipase from *Lactobacillus plantarum* - In International Symposium on "Proteomics: Present and Future" 2014, CCMB, Hyderabad, India, Nov 22-24.
3. **U. Sita Ramyasri** and Jayati Ray Dutta: Lipase mediated ester synthesis from different lactic acid bacteria through co-culture technique - In International Conference on Biodiversity, Bioresources and Biotechnology, 2014, Mysore, India, Jan 30-31.
4. **U. Sita Ramyasri**, Harshitha and Jayati Ray Dutta: Optimization of culture conditions for the production of lipase by co-culture of *Lactobacillus brevis and Lactococcus lactis* - In International Conference on Advances in Chemical Engineering (ICACE, 2013), NIT, Raipur, Chhattisgarh, India, April 5-6.

5. **U. Sita Ramyasri** and Jayati Ray Dutta: Statistical optimization of culture conditions by Response surface methodology, Artificial neural network and Genetic algorithm for synthesis of lipase from *Lactococcus lactis* – In World Congress Biotechnology, Bright International Conference, 2012, Leonia International Convention Centre, Hyderabad, India, May 4-6.
6. **U. Sita Ramyasri** and Jayati Ray Dutta: Enzymatic transesterification of non-edible oils for the production of biofuels – In contemporary trends in Biological and Pharmaceutical Research (CTBPR-2011) Conference, 2011, BITS-Pilani, Rajasthan, March 12-13.

Workshops attended:

1. Workshop on Research Methodology – In Centre of Excellence in Water Resources Management, Department of Civil Engineering, BITS-Pilani, Hyderabad, April 11-12, 2014.

Awards:

1. Best Poster Presentation Award in the International Conference on advances in Chemical Engineering (ICACE - 2013), NIT, Raipur, April, 5-6, 2013.
2. Special Jury Award for oral presentation on "Lipase mediated ester synthesis from different lactic acid bacteria through co-culture technique" in the International Conference on Biodiversity, Bioresources and Biotechnology, Mysore, India, Jan 30-31, 2014.

Manuscript communicated:

1. Expressed and immobilized lactic acid bacterial lipase for synthesis of flavour esters: Sita Ramyasree Uppada, Mahesh Akula, Anupam Bhattacharya and Jayati Ray Dutta, communicated to 3 Biotech Journal, Springer (under review).