Screening and evaluation of lipase from extremophilic bacteria: A study on kinetic and thermodynamic characteristics

THESIS

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CERTIFICATE

This is to certify that the thesis titled "***Screening and evaluation of lipase from extremophilic bacteria: A study on kinetic and thermodynamic characteristics***" submitted by Ms. Kanchan Sonkar is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university. The thesis submitted to Babasaheb Bhimrao Ambedkar University Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D.) regulations -1999 as amended in 2008/2010/2013 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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DECLARATION

I hereby declare that the thesis entitled ***"Screening and evaluation of lipase from extremophilic bacteria: A study on kinetic and thermodynamic characteristics"*** is my own work conducted under the supervision of Prof. Naveen Kumar Arora, Department of Environmental Science, at Babasaheb Bhimrao Ambedkar University, Vidya Vihar, Raebareli Road, Lucknow, and is also approved by Departmental Research Committee (DRC). I further declare that to the best of my knowledge, the thesis does not contain any part of work, which has been earlier submitted for the award of any other degree either in this university or in any other University/ Deemed University.

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PREFACE

Extremophiles are a group of organisms that thrive under extreme environmental conditions in which most of the life forms could not survive. These extreme environmental conditions can be due to temperature, pH, salinity, and pressure. Extremophiles are comprised of animals, plants, insects, fungi, and bacteria. Extremophilic microorganisms can be described as acidophilic (low pH condition), alkaliphilic (high pH), halophilic (high salt concentrations), thermophilic (high temperature), hyperthermophilic (optimally survive extreme high temperature usually above 80°C), psychrophilic (survive at low temperature), piezophilic/barophilic (optimized growth at high pressure), oligotrophic (grow in nutrient deficient environments), endolithic (grow within rock spaces), and xerophilic (thrive in dry area). Extremophiles have gained immense interest for industrial purpose due to their ability to catalyze reactions under severe conditions. Their enzymes have evolved to be functional under such extreme conditions, where their mesophilic counterparts can not even survive, providing additional features of high value for industrial process development.

Extremophilic lipases have different combinations of substrate specificity, low and high operative temperature and a wide range of pH, thermostability, tolerance to organic solvents and activity under the presence of different inhibitors, surfactants and metal ions. These characteristics of lipase make it most valuable enzyme for industrial biocatalyst. Thus, the production of extremophilic bacterial lipase in different environmental conditions could be a new approach and may be an area of interest for production of many new natural products which get modified or produced in excess under extreme environmental conditions. The present study is an attempt to isolate the extremophilic bacteria and study their enzyme production and activity under different environmental conditions such as temperature, pH, salinity, surfactants, inhibitors and metals ions. The extracellular lipase produced by

psychrotolerant bacterial strain was compared with that of halotolerant strains. It was postulated that both lipase enzymes possessed different characteristics. The kinetic and thermodynamic study of lipase was done by Michelis-Menton and Lineweaver-Burk plot.

The extremophilic bacteria were also used to study biodegradation of Di(2-ethylhexyl phthalate) with the help of UV-Vis absorption spectrometry, gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC).

Chapter 1, 'Introduction' gives the outline of the present work along with the list of objectives of study to be achieved.

Chapter 2, 'Review of Literature' is related with the review and citation of the work done by other investigators working in the area of lipase production, and its activity under extreme environmental conditions.

Chapter 3, 'Isolation, Screening, Biochemical characterization of extracellular lipase producing extremophilic bacterial strains' is regarding isolation of bacterial strains, their morphology and biochemical characterization.

Chapter 4, 'Kinetic and thermodynamic study of extracellular lipases isolated from extremophilic bacterial strains' deals with the kinetic and thermal properties of both bacterial strains associated with change in the secondary structure of lipase at different temperatures.

Chapter 5, 'Biodegradability and biodegradation pathways of Di(2-ethylhexyl) phthalate by two extremophilic bacterial strains' deals with the biodegradation of DEHP at different temperature and pH by both the bacterial strains.

Chapter 6 'General Discussion' with critical analysis of the various parameters followed by 'Summary and Conclusion'.

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NOMENCLATURE

ABBREVIATIONS

16S rRNA	16S ribosomal ribonucleic acid
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
BME	2-mercaptoethanol
CD	Circular dichroism
CTAB	cetyl trimethylammonium bromide
DEHP	Di(2-ethylhexyl) phthalate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
GC/MS	Gas chromatography-mass spectroscopy
HPLC	High performance liquid chromatography
KCl	Potassium chloride
MBP	Monobutyl phthalate
MEHP	Mono-(2-ethylhexyl) phthalate
NA	Nutrient agar
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information

OD	Optical density
PA	phthalic acid
PCA	Protocatechuic *acid*
PMSF	phenylmethylsulfonyl fluoride
pNP	p-Nitrophenyl
pNPP	p-Nitrophenyl palmitate
ppm	Parts per million
Rf	Retention factor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning Electron Microscopy
T-20	Tween 20
TBA	Tributyrin agar plate
T-X 100	Triton X 100
UV	Ultra Violet radiations
UV-Vis	Ultra Violet-Visible
Viz.	Videlicet

SYMBOLS

kDa	kilo Dalton
M	Molar

mm	Millimetre
cm	Centimetre
g	Gram
rpm	Rotation per minute
hr	Hour
gm/L	Gram per litre
psi	Per square inch
mM	Millimolar
μL	Microlitre
nm	Nanometer
ε	Molar extinction coefficient
mdeg	Millidegree
mg/L/h	Milligram per liter per hour
μm	Micrometer
μ	Micron
°C	Degree Celsius
v/v	Volume/volume
w/v	Weight/volume
C_0	Initial enzyme activity
C_t	Enzyme activity at time t
D	Decimal reduction time
Ea	Activation energy
$E_{a(d)}$	Inactivation energy
h	Plank’s constant

J/K	Joule/kelvin
k	Rate constant
k_b	Boltzmann’s constant
k_{cat}	Catalytic constant
k_d	Deactivation rate constant
K_m	Michaelis constant
R	Gas constant
R_m	Rate of substrate transformation
S	Substrate concentration
S_0	Initial substrate concentration
T	Absolute temperature
t	Incubation time
$t_{1/2}$	Half-life
V_{max}	Maximum velocity
ΔG^*	Gibb’s free energy
ΔH^*	Enthalpy
ΔS^*	Entropy

CHAPTER 1

CHAPTER 1
GENERAL INTRODUCTION

1.1 Introduction

Enzymes are generally proteins of high molecular weight and they act as a catalyst in the living organisms. An enzyme catalyzes a chemical reaction without itself being altered in the process. Though some RNA molecules are also known to act as a catalyst but the majority of cellular reactions are regulated by protein catalysts. Enzymes are specified, versatile biological catalysts which stimulate the rate of reaction as compared to chemical catalysts (Shuler and Kargi, 2002). Enzymes have been used since the ancient civilization and as of today, almost 4000 enzymes have been discovered. Near about 200 enzymes are being used in the industrial and commercial purposes. Most of the known enzymes are produced from microbial origin. An increased number of enzymes are being produced industrially to meet the demand at affordable cost which are used in fermentation processes, recovery methods and biochemical research.

Furthermore, due to numerous alterations, enzymes can catalyse a number of substances used in industrial applications (Sharma et al., 2001). Enzymes are substrate specific and are classified into six major classes by International Union of Biochemists (IUB), depending upon the reaction type. Enzymes belong to six classes; oxidoreductases, hydrolases, transferases, lyases, isomerases, ligases.

Lipases are triacylglycerol acylhydrolases belonging to E.C. 3.1.1.3 classification, which are ubiquitous and may be considered for industrial potential due to physiological significance. Lipases catalyze the hydrolysis of triacylglycerols into glycerol and free fatty acids. Lipases are serine hydrolases which are defined as triacylglycerol acylhydrolases (E.C. 3.1.1.3). Lipases are different from esterases enzyme (E.C. 3.1.1.1) by the nature of

their substrates. Lipases are active only when adsorbed to an oil–water interface rather than esterases (Martinelle et al., 1995) and do not hydrolyze dissolved substrates in the bulk fluid. Carboxylester hydrolases, usually named as esterases, include a wide range of enzymes, which are defined due to their ability to catalyze the hydrolysis of carboxylic ester bonds and are largely distributed among different living organisms, plants, and microorganisms. Lipases are lipolytic enzymes belonging to a special class of carboxylic esterases which are capable of releasing long-chain fatty acids from natural water-insoluble carboxylic esters. They show chemioselectivity, enantioselectivity and regioselectivity characteristics. Lipases exhibit wide substrate specificity and a wide range of optimum temperatures for their activity. All these properties of lipases make them one of the most versatile biocatalysts for industrial applications (Kademi et al., 2005). The terms lipases and lipolytic enzymes include two main groups of enzymes, carboxylesterases and true lipase, which differ from each other with respect to their preference for long and short chain substrates, respectively. Other differences are listed in the Table 1.1 (Barros and Fleuri 2010).

Table 1.1 Differences between true lipases and carboxyl esterase

PROPERTY	TRUE LIPASES	CARBOXYL ESTERASE
Substrate	Long chain triglycerides esters	Short chain triglycerides esters
Substrate range	Broad	Broad to tight
Regio and stereo selectivity	High	High to low
Stability and organic solvent activity	High	High to low
Substrate physical state	Insoluble	Soluble
Substrate hydrophobicity	High	High to low
Scissile fatty acid binding site	Long	Short

Interfacial activation	Yes	No
Optimum pH	8-9	5.5-7

Bacterial lipolytic enzymes are differentiate by their capability to catalyze a wide range of chemical reactions. Lipase assay are analyzed by different methods used as p-nitrophenyl hydrolysis, the pH-stat method and the monolayer technique that enable the comparison between the results on substrate specificities. Previous reports are available to standardize the measurements and comparative studies of lipase assay (Rogalska et al., 1993, Simons et al., 1998). Among different types of microbes only some bacterial lipases have been investigated so far (Svendsen et al., 1995).

Lipases are extensively distributed in different microorganisms such as bacteria, fungi, plants and living animals (Bornscheuer 2002; Hasan et al., 2006). The study of hydrolysis of triglycerides by enzymes started over 300 years back and the lipases ability to catalyze the hydrolysis and synthesis of esters have been recognized about 70 years ago (Eijkman 1901). Claude Bernard discovered the first lipase in pancreatic juice in 1856 (Hasan et al., 2006). Lipase was initially discovered from animal pancreas and was used as a digestive aid. Due to shortage of animal pancreas, animal pancreatin was replaced by lipases from plant seeds and microbes. At present commercial lipases are mainly produced by microbes (Barros and Fleuri 2010). Microbial enzymes are always preferred than their counterparts from animals and plants due to their wide range of catalytic activities. The microbial enzyme supply is not affected by the seasonal fluctuations. The production of microbial enzymes is safe, stable and convenient as well as the microbes produce them at a high rate in inexpensive media. Genetic manipulation of lipase enzyme can be easily done in microbes as compared to plants and animals (Wiseman 1995).

The production of microbial lipase is influenced by the culture conditions. Optimization of culture conditions enhances the productivity of enzyme, which influences the enzyme

producers and the ratio of the extracellular to intracellular lipases. The quantity of lipase produced is dependent on numerous physico-chemical and environmental factors such as pH, temperature, carbon source, lipid sources, nitrogen source, concentration of inorganic salt, presence of inducers, surfactants, and the availability of oxygen. The additional substrate such as olive oil, butter, lard, and fatty acid stimulate the in vitro lipase production (Omar et al., 1987). Although, the study of lipase production has been performed for many years and large-scale enzyme production is possible by the microbial growth in a fermenter at a large scaler, the use of microbial lipases is confined mainly to the process of oleo-chemistry and production of dairy based industries.

Generally, microbial lipases are extracellular in nature (Prabaningtyas et al., 2018). The extracellular lipases are reported to be produced by *Bacillus* sp. (Sugihara et al., 1991; Sidhu et al., 1998a, Sharma et al., 2001, Balaji et al., 2020); *Pseudomonas* sp. (Lin et al., 1995; Gupta et al., 2006, Khosla et al., 2017), *Rhizopus stolonifer 1aNRC11* (Mohamed et al., 2021), *Mucor hiemalisfhiemalis* (Hiol et al., 1999), *Beauveria bossiana* (Hegedus and Khachatourians, 1988), *Penicillium* sp.(Lima et al., 2019), *Rodotorula pilmanae* (Muderhwa et al.,1986), *Humicola* sp. (McPherson et al., 2020, Omar et al., 1987), *Candida* sp. (Monteiro et al., 2021, Guo et al., 2020). It has been observed in the last quarter of the 20th century, the application of microbial lipases has increased for different commercial purpose such as pharmaceuticals, synthesis of pesticides, production of single cell protein, production of cosmetics, waste disposal management and biosensor modulations (Pandey et al., 1999; Jaeger and Reetz 1998; Berglund 2001; Yamne et al., 2002). Some of the microbial lipase producers appear to synthesize more than one lipase (Molinari et al., 1996; Gupta et al., 2006). The mixture of lipase(s) may tend to have different biochemical and catalytic properties than a pure lipase.

1.2 Catalytic mechanism of lipase

Lipases are versatile in nature as they can catalyze a broad range of chemical reactions as ester hydrolysis and synthesis, esterification, transesterification and intertransesterification. As shown in Fig 1.1, lipase hydrolyses triacylglycerols and other esters in aqueous medium. In the non-aqueous medium or in organic solvents, lipase catalyze the reverse reaction of ester synthesis or esterification and exchange of acyl groups between triacylglycerides, alcohols, esters, glycosides and amines (Schmidt 1999; Bornscheuer 2002; Gupta et al., 2004a).

Some specific characteristics of extracellular lipases are listed as follows:

- Substrate specific: Lipase exhibits a different range of specificity for various substrates (Pandey et al., 1999).
- Regiospecific: Lipase shows positional preference for the ester bond hydrolysis which is responsible for the formation of free fatty acids, mono and di -acyl glycerols (Gunstone 1999).
- Fatty acid specific: Lipase show preference for acyl chains they hydrolyse based on length of chain, position, number and configuration of double bonds, presence of branched groups and the nature of acyl source (Gunstone 1994).
- Enantio/Stereoselective: Lipase distinguish the enantiomers present in racemic mixture and is an important property employed in organic synthesis (Muralidhar et al., 2002; Gupta et al., 2004a).

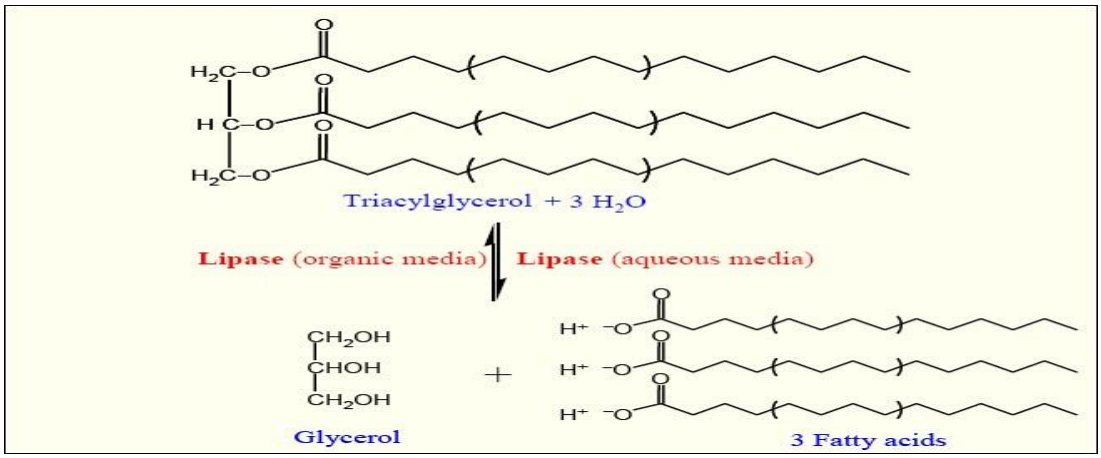


Figure 1.1 Catalytic activity of lipase enzyme in aqueous and non-aqueous media

Table 1.2 List of some lipase producing bacteria

MICROORGANISMS	REFERENCES
Acinetobacter sp.	Wakelin and Forster (1997), Barbaro et al., (2001), Snellman et al., (2002)
Acinetobacter radioresistens	Li et al., (2005); Liu and Tsai (2003)
Acinetobacter calcoaceticus	Dharmsthiti et al., (1998), Jaeger et al., (1999), Pratuamgdejkul and Dharmsthiti (2000)
Aeribacillus pallidus strain VP3	Ktata et al, (2020)
Arthrobacter sp.	Pandey et al., (1999)
A. fulgidus	Jaeger et al., (1999)
Bacillus sp.	Sidhu et al., (1998a), (1998b), Pandey et al., (1999), Sharma et al., (2001), Nawani and Kaur (2000), Ertugrul et al., (2007)
Bacillus sp. VITL8	Balaji et al., (2020)
Bacillus sp.	Lee et al., (2015)
B. atrohaeus	Ameri et al., (2019)
B. stearothermophilus	Fernandes et al., (2007); Bradoo et al., (1999) Jaeger et al., (1999)
B. coagulans	Alkan et al., (2007), Shafei and Rezkallah (1997)
B. subtilis	Takaç and Marul (2008), Ruiz et al., (2005) Eggert et al., (2003), Jaeger et al., (1999)

B. subtilis AKL13	Nathan & Rani (2020)
B. subtilis B10	Kuppamuthu et al., (2021)
B. amyloliquefaciens E1PA	Saengsanga et al., (2016)
B. atrophaeus	Bradoo et al., (1999)
B. subtilis PCSIRNL-39	Mazhar et al., (2017)
B. pumilus	Jaeger et al., (1999)
B. thermocatenulatus	Jaeger et al., (1999)
Burkholderia glumae PG1	Voget et al., (2015)
B. cereus	Vasiee et al., (2016)
B. cereus	Hassan et al., (2018)
B. licheniformis KM12	Malekabadi et al., (2018)
B. methylotrophicus PS3	Sharma et al., (2017)
B. stratosphericus L1	Gricajeva et al., (2016)
B. thermoleovorans	Rua et al., (1997)
B. thermosphacta	Brune and Gotz (1992)
B. glumae	Jaeger and Reetz (1998)
B. cepacia	Fernandes et al., (2007)
B. multivorans	Gupta et al., (2007)
B. subtilis I-4	Iqbal and Rehman (2015)
Chromobacterium viscosum	Jaeger and Reetz (1998), Jaeger et al., (1999)
Corynebacterium acnes	Brune and Gotz (1992)
E. faecalis	Kar et al., (1996)
Geobacillus thermoleovorans DA2	Abol et al., (2016)
Geobacillus thermodenitrificans AV-5	Christopher et al., (2015)
Lactobacillus plantarum	Lopes et al., (2002)
Lysinibacillus sphaericus	Aderiye & Sulaimon (2017)
Marino bacterlipolyticus	Oves et al., (2017)
Microthrix parvicella	Wakelin and Forster (1997)
Moraxella sp.	Jaeger et al., (1999)
Mycobacterium chelonae	Pandey et al., (1999)
Pseudomonas sp.	Kiran et al., (2008); Sarkar et al., (1998)

P. aeruginosa	Ruchi et al., (2008); Mahanta et al., (2008)
P. monteilli	Rasmey et al., (2017)
P. otitidis G5	Haque et al., (2019)
P. luteola	Arpigny and Jaeger (1999)
P. mendocina ED9	Sankar& Achary (2020)
P. mendocina	Jaeger et al., (1999)
P. wisconsinensis	Arpigny and Jaeger (1999)
Pseudomonas sp. ISTPL3	Khosla et al., (2017)
Planomicrobium sp. MR23K	Teymouri et al., (2018)
Psychrobacter immobilis	Jaeger et al., (1999)
Pasteurella multocida	Pratt et al., (2000)
Propionibacterium acnes	Jaeger et al., (1999)
Propionibacterium avidium	Brune and Gotz (1992)
Propionibacterium granulosum	Brune and Gotz (1992)
Proteus vulgaris	Jaeger et al., (1999)
Staphylococcus hominis	Behera et al., (2019)
S. hyicus	Jaeger et al., (1999)
S. haemolyticus	Oh et al., (1999)
S. aureus	Gotz et al., (1998); Simons et al., (1996)
S. capitis SH6	Rmili et al., (2021)
S. warneri	Talon et al., (1995)
S. xylosus	Mosbah et al., (2005)
Serratia rubidaea	Immanuel et al., (2008)
Sphingobacterium sp. strain S2	Satti et al., (2019)
Thalassospira permensis	Kai and Peisheng (2016)

1.3 Classification of bacterial lipases

Enzymes were classified based on their substrate specificity and condition of chemical reaction or by similarity of amino acid sequence. The first method of classification is very difficult as the enzymes are isolated worldwide using different substrates and reaction conditions at various laboratories. On the other hand, increasing availability of amino acid sequence of enzyme in public databases (PDBs) allows comparison which gives a clear idea about the resemblance and evolutionary relationships between different type of lipases, but it cannot be correlated with the enzymatic properties (Bornscheuer 2002).

Lipases were classified by Arpigny and Jaeger (1999) into eight families based on the conserved sequence motifs and biological properties. Out of them, 53 lipases and esterases, which were further extended and classified by Nthangeni et al. (2001); Jaeger and Eggert (2002). The classification of bacterial lipase is presented in Table1.2 (Arpigny and Jaeger 1999).

FAMILY I

Most of the Gram-positive and Gram-negative bacteria producing extracellular lipase belong to Family I. The extracellular lipase from Family I are generally considered as true lipases, which are further divided into seven sub-families. Sub-families I.1, I.2 and I.3 contain lipases from *Pseudomonas* sp. and other Gram-negative bacteria of 30–32 kDa molecular weight. Lipases belong to subfamilies I.1 and I.2 consist a signal peptide at N-terminal to facilitate their excretion via secretion pathway type II (Sec–Xcp system). Whereas sub-family I.3 enzymes do not have a signal peptide and are secreted by type I pathway via ABC transport. Subfamilies I.4 and I.5 contain extracellular lipase from *Bacillus* sp. and *Geobacillus* sp. (Jozo 1988).

Lipases from the mesophilic *Bacillus* sp., which consists of smallest lipases known (~20 kDa), are assigned in subfamily I.4. Lipases from *Geobacillus* sp. are thermotolerant and alkalophilic in nature which possess ~45 kDa molecular weight protein and exhibit maximum activity at 9.0 pH and 65°C temperature, are grouped in sub-family I.5 (Zottig et al., 2017).

The extracellular lipase from *Staphylococcus* belongs to Sub-family I.6. This family includes lipases which contain ~75 kDa molecular weight, secreted as precursors and cleaved in the medium by a specific protease. A specific chaperone facilitates the folding and translocation of the enzyme through the cell membrane (Hitch et al., 2019). Lipase from *Staphylococcus hyicus* also possess notable phospholipase activity, which is considered exclusive among true lipases. Sub-family I.7 include *Propionibacterium acnes* and *Streptomyces cinnamoneus* lipases, which show substantial similarity among them. Their dominant region exhibits ~50% similarity with subfamily I.2 and I.4 (Arpigny and Jaeger 1999).

FAMILY II (GDSL FAMILY)

Lipases categorized under family II (GDSL) do not display the usual pentapeptide Gly-Xaa-Ser-Xaa-Gly to house the nucleophilic serine; instead they have a Gly-AspSer-(Leu) [GDS (L)] motif which is nearer to the N-terminus compare to other lipases. Interestingly, esterase from *Streptomyces scabies* contains a Ser-His catalytic dyad and a specific tertiary fold (Arpigny and Jaeger 1999).

FAMILY III

Family III is comprised of lipases from a psychrophilic *Moraxella* sp. and from numerous *Streptomyces* sp. Family III was first identified and cited by Wei et al. (1998) who explained the 3D structure of the *Streptomyces exfoliates* (M11) lipase.

FAMILY IV (HSL FAMILY)

Lipases of family IV display amino-acid sequence blocks that show resemblance with mammalian lipases. These sequence blocks are considered to be responsible for cold adaptation. This family of lipases also includes mesophilic and thermophilic enzymes (Bornscheuer 2002).

FAMILY V

Lipases included in family V are produced by psychrophilic and mesophilic microorganisms. They show significant amino acid sequence resemblance (20–25%) with other enzymes of bacteria which contain the distinctive α/β-hydrolase fold and the catalytic triad like epoxide hydrolases, haloperoxidases and dehalogenases.

FAMILY VI

Family VI comprises of the smallest carboxylesterases with 23–26 kDa molecular weight that showed ~40% sequence resemblance with eukaryotic lysophospholipases

FAMILY VII

Enzymes grouped in family VII are large esterases (50–65 kDa) which share significant amino acid sequence resemblance with eukaryotic acetylcholine esterases and liver or intestinal carboxylesterases.

FAMILY VIII

Family VIII consists of enzymes which contain ~380 residues that show a prominent similarity with some class C β-lactamases. Two of them have the conventional Gly-Xaa-Ser-Ala-Gly motif, which is not followed by histidine and located near the C terminus of the protein. Studies on site directed mutagenesis revealed that the catalytic serine is not

located in this pentapeptide, but in a Ser-Xaa-Xaa-Lys motif present at N-terminal region (Bornscheuer 2002).

Table 1.3 Classification of bacterial lipases

Family	Subfamily	Enzyme producing strain	Properties
I	1	*P. aeruginosa*	True lipases
		P. fluorescens	
		V. cholera	
		Acinetobacter calcoaceticus	
		P. fragi	
		P. wisconsinensis	
		Proteus vulgaris	
	2	*Burkholderia glumae*	
		Chromobacterium viscosum	
		Burkholderia cepacia	
		P. luteola	
	3	*P. fluorescens*	
		Serratia marcescens	
	4	*B. subtilis*	
		B. pumilus	
	5	*B. stearothermophilus*	
		B. thermocatenulatus	
	6	*Staphylococcus hyicus*	Phospholipase
		Staphylococcus aureus	
		Staphylococcus epidermidis	
II (GDSL)		*Aeromonas hydrophila*	Secreted acyltransferase
		Streptomyces scabies	Secreted esterase
		P. aeruginosa	Esterase
		Salmonella typhimurium	Esterase
		Photorhabdus luminescens	Secreted esterase

III	*Streptomyces exfoliates*	Extracellular lipase
	Streptomyces albus	Extracellular lipase
	Moraxella sp.	Extracellular esterase 1
IV(HSL)	*Alicyclobacillus acidocaldarius*	Esterase
	Pseudomonas sp. B11-1	Lipase
	Archaeoglobus fulgidus	Carboxylesterase
	Alcaligenes eutrophus	Putative lipase
	E. coli	Carboxylesterase
	Moraxella sp.	Extracellular esterase 2
V	*P. oleovorans*	PHA-depolymerase
	Haemophilus influenza	Putative esterase
	Psychrobacter immobilis	Extracellular esterase
	Moraxella sp.	Extracellular esterase 3
	Sulfolobus acidocaldarius	Esterase
	Acetobacter pasteurianus	
	Spirulina platensis	
	P. fluorescens	
	Rickettsia prowazekii	
	Chlamydia trachomatis	
VII	*Arthrobacter oxydans*	Carbamate hydrolase
	B. subtilis	p-Nitrobenzyl esterase
	Streptomyces coelicolor	Putative carboxylesterase
VII	*Arthrobacter globiformis*	Stereoselective esterase
	Streptomyces chrysomallus	Cell-bound esterase
	P. fluorescens	Esterase III

Lipases are surface-active enzymes due to their binding specificity to emulsified triglyceride substrate which significantly increases their hydrolytic activity as compared to their activity towards a dissolved substrate (Liebeton et al., 2001). They have capability to catalyze and synthesize esters from glycerol and long chain fatty acids. These chemical reactions usually proceed with higher regio and or enantio-selectivity, thus making microbial lipase an important biocatalyst in organic chemistry. However, lipases are activated only when adsorbed to an oil-water interface (Martinelle et al., 1995). A true lipase can split emulsified esters of glycerol and long fatty acids such as triolein and tripalmitin. The large number of lipases with appropriate characteristics are becoming available and efforts are being made to commercialize biotransformation and synthesize based on these lipases (Reetz and Jaeger 1998).

Microbial lipase are presently attracting enormous attention because of their industrial and biotechnological applications (Jaeger and Reetz et al., 1998). Most of the lipases used for industrial purpose belong to fungal and bacterial origin. Among bacterial lipases, main attention was given on some specific classes such as the lipases from the genus *Pseudomonas,* which are specifically used for biotechnology application (Gilbert et al., 1993 and Svendsen et al., 1995). Inappropriately, there is partial and sporadic information on the connection of the many bacterial lipases and esterase. Some biochemical properties of extracellular lipases as pH, temperature and the activity in presence of Ca^{+2} ions have been studied in genera *Bacillus, Pseudomonas* and *Staphylococcus* (Gilbert et al., 1993 and Svendsen et al., 1995, Gotz et al., 1998).

Microbial lipase plays a significant role in the food industry and are used in the processing of a polyunsaturated fatty acid (PUFA), named as g-linolenic acid, and are also used in the production of methyl ketones, a flavour molecule characteristic of blue cheese. Microbial lipase is used in the production of 4-hydroxydecanoic acid (precursor of g-decalactone),

dicarboxylic acids (a fruit flavour) used as prepolymers and interesterification of inexpensive glycerides to more valuable forms (Undurraga et al., 2001). It is helpful in the modification of vegetable oils to synthesize fats and human milk fat for use in baby feeds; isopropyl myristate a lipid has been used in cosmetics products; and some monoglycerides used as emulsifiers in pharmaceutical and food applications. The growing consciousness of the significance of chirality in the perspective of biological activity has encouraged a large demand for effective methods for pure enantiomers production. Microbial lipase helps in the formation of enantiomers, which work as chiral anti-inflammatory drugs as naproxen and ibuprofen (Xin et al., 2001, Lee et al, 1995; Arroyo et al., 1995). It also contributes to the formation of hypertension medicine due to consistence of antihypertensive agents as angiotensin that convert enzyme (ACE) inhibitors such as captopril, enalapril, ceranopril, zofenapril, and lisinoprii and diitiazem – a drug that blocks calcium channel (Berglund and Hutt, 2000). Microbial lipases have been also used to catalyze the synthesis of variety of esters of sugars and carbohydrates that are used as neutraceuticais and additives in cosmetics (Berglund, 2001). Production of various natural esters and modified triglycerides by esterification and transesterification reactions has been achieved using the microbial lipases (Gabriela et al., 2003). Bacterial lipases have numerous applications in industries and biotechnology as describe below in Table 1.4.

Table 1.4: Type of reaction and commercial applications of lipases obtained from bacterial sources

REACTION TYPE	SOURCE	COMMERCIAL APPLICATIONS	SUBSTRATE	REFERENCES
Hydrolysis	*B. pumilus*	Food and detergent, formation of bioorganic compounds	Long chain triacylglycerols	Laachari et al., 2015
	B. licheniformis	Oil production industry	Trimyristin	Rashid et al., 2013

	B. thermoleovorans	Oil industry	Triglycerides and oil	Patil et al., 2011
	Propionibacterium acnes	Food industry	Triolein	Jaeger-Eggert et al., 2002
	P. aeruginosa	Treatment of solid waste	Phorbol 12 myristate 13-acetate	Jaeger-Eggert et al., 2002
	S. aureus	Medical industry	Trilinolein	Bose et al., 2013
	Acinetobacter radioresistens	Chemical industry	4-nitrophenyl caprylate	Thakur 2012
	B. licheniformis	Healthcare product	Coumaric acid Gallic acid	Sharma and Kanwar 2014
	S. epidermidis	Food industry	Fatty acid and alcohols	Patil et al., 2011
Esterification	*Acinetobacter*	Flavour industry	Caprylic acid + ethanol	Sun et al., 2012
	B. subtilis	Biodiesel	Waste cooking oil	Treichel et al., 2010
	Burkholderia cepacia	Biodiesel, hydrocarbon	Jatropa oil	Bajaj et al., 2010
	Chromobacterium viscosum	Biodiesel	Jatropa oil	Bajaj et al., 2010
	E. aerogenes	Hydrocarbon oil	Jatropa oil	Bajaj et al., 2010
	Geobacillus sps.	Biodiesel production	Vegetable oil	Christopher et al., 2015
	Pseudomonas sps.	Biodiesel	Soyabean oil	Cesarini et al., 2015
Transesterification	*S. haemolyticus*	Biodiesel production	Olive oil and methanol	Kim et al., 2013
	B. stearothermophilus	Production of different fatty acids.	Tripalmitin	Thakur 2012
	P. fluorescens	Food industry	Black currant oil	Patil et al., 2011
Alcoholysis	*Pseudomonas sps.*	Biodiesel	Triglycerides	Salis et al., 2009

Enantioselective Hydrolysis	*Serratia marcescens*	Medical and health care product	(±)-*trans*-3-(4-methoxyphynyl) glycidic acid methyl este	Su et al., 2014

1.4 Current demand of lipase

Hydrolases group of enzymes such as proteases, lipases and carbohydrases constitute >95% of the total enzyme production. Proteases and carbohydrases have been used by industries for many years and they have consisted the largest share in the world enzyme production market (Vakhlu and Kour, 2006). Although, lipases alone account for 5–10% of the total world enzyme market, but considering their extraordinarily high flexibility with different enzyme substrates they are in demand more than any other enzyme (Gandhi, 1997). Recently, lipases have arisen as key enzymes in biotechnology and pharmaceutical industry due to their flexibility and catalytic properties, which can be useful for a wide range of applications (Gupta et al., 2004). The present scenario of industrial and biotechnological application of microbial lipase shows that it is useful in the manufacturing of pharmaceutical products, pesticides, biodiesel, biosensor production and waste management (Villeneuve et al., 2000; Vakhlu and Kour, 2006) techniques. Microbial lipase enclosed with several specific characteristics make them highly desirable biocatalysts for commercial purpose. These characteristics include catalytic function on the basis of molecular basis structure, which makes it a model to study the regulation of interfacial enzyme-catalyzed reactions. The efficiency of lipase as a biocatalyst for synthesis of product via esterification, transesterification, aminolysis in organic solvent and their substrate specificity surpasses it contribution in different types of industrial applications (Yan et al., 2018). Lipases are broadly used in the fats and oil processing, formulation of detergents, food processing, synthesis of pharmaceuticals products, manufacturing of paper

and cosmetics production (Rubin and Dennis, 1997a,b). Microbial lipases can also be used in the degradation of fatty waste (Masse et al., 2001) and polyurethane (Takamoto et al., 2001).

1.5 Industrial applications

Lipases are steadily switching chemical reactions as they are eco-friendly and low-cost products. Microbial lipases are produced less methods which reduce environmental pollution and provide value added products. Thus, global enzymes market is looking for its new applications. Use of lipase in many different industries are mentioned here:

Food industry

Microbial lipase is used as a catalyst to release the fatty acids by hydrolysis of oils and fat in food products (Barros et al., 2010). The exceptional properties of selectivity and specificity of lipase help in the synthesis of many food products such as fruit juices, bakery foods, vegetable processing, dairy product fermentation and enrichment. Microbial lipase has capability to remove fat from fish and meat products as well as it enables the biotransformation of complex materials (Sharma et al., 2001). Phospholipase is also use for production of mayonnaise from egg yolk and lecithin modification. The major commercial application of lipase is their use in hydrolysis of fat and oils. The production of dairy products such as fat and oil modification, cheese formation, butter and margarine production and baby food, truly depend on the lipase use. The formulated lipids with exclusive properties such as cocoa butter work as human milk substitute rich in poly unsaturated fatty acids (PUFA) and enriched oils are also made by using lipase. The flavour of cheese is enhanced by lipase catalysis incubating at high temperature. The enzyme modified cheese (EMC) is useful in the making of dips, soups, snacks and sauces (Aravindan et al., 2007).

The lipases are used as emulsifiers in the bakery products to improve the flavour for animal feeding. They are also used to increase the flavours and fragrance of food products. Enzyme based technology has been used for improvement of some fewer desirable fats to cocoa butter substitutes (Undurraga et al., 2001). This technology of immobilization of *Rhizomucor miehei* lipase was used to replace the palmitic acid in palm oil with stearic acid by transesterification. Similarly, Pabai et al., (1995) described a technique of interesterification of lipase to reduce the long-chain saturated fatty acid in butter fat which resulted into equivalent increase in C18:0 and C18:1 acid at position 2 of the selected triacylglycerol.

Lipases have been used in food supplement industry for the synthesis of L-ascorbic acid fatty acid esters, low-calorie triacylglycerol and antioxidants, tocopherols (vitamin E) etc. (Ferreira et al., 2002). Lipase from *Bacillus subtilis* used in the bakery industry increases the volume of loaf in wheat bread, which is influenced by the endogenous lipid. Microbial lipases are widely used in the formulation of lipolysed milkfat that increase the consistency and aroma of milk (Jooyandeh et al., 2009, Tambe et al., 2015). Some other commercially available lipases such as Palatase 20000 L, Calf PGE and AY30G have been developed to increase the flavour profile of milk. Bacterial lipases from *Staphylococcus warneri*, *Staphylococcus xylosus*, *Mucor miehei* and *Candida antarctica* were used to enhance flavour in immobilized form in bakery and food processing industries (Sharma & Kanwar 2014).

Detergent industry

Microbial lipases are used in the detergent industry in combination with both proteases and amylases, to improve the efficacy of detergents. Lipases adsorbsed on to the surface of fabric and create a stable complex of fabric-lipase which catalyzes the chemical bonds upon

adding of water (Hasan et al., 2006). In the detergent industries there is a great demand of thermophilic, alkaliphilic, water soluble lipase which exhibit low substrate specificity and are tolerant to detergent and other chemical surfactants that are compatible to use with washing powders as well as liquid detergents (Carvalho et al., 2015).

It is found that the chelating agents available in commercial detergents leads to inactivation of enzyme, however in the case of *B. licheniformis* lipase, calcium chloride is added to the enzyme detergent complex to restore the activity of enzyme (Romdhane et al., 2010). Novel lipases from *Talaromyces thermophilus, Pseudomonas aeruginosa* and other bacterial sources have been investigated and they are found to have potential use in the detergent industry (Kanjanavas et al., 2010, Grbavčić et al., 2011). Extracellular lipase from *Pseudomonas fluorescens* immobilized onto a woolen cloth was found to be capable of removing oil stains. The immobilization of lipase has a unique procedure involving chlorination of the wool that improves the activity of lipase.

Detergent industry is the largest market in industrial and commercial enzymes production (Ahuja et al., 2004). Microbial lipase possess ability to improve the washing efficiency of detergents towards the fatty food stains from such fabrics which are difficult to wash during normal washing conditions (Andree et al., 1980). Microbial lipases showed ideal detergent condition, which include stability at high pH and active in the presence of different surfactants (Jurado et al., 2007). Lipases should work as oxidizing and chelating agents that may be useful in detergents as active oxygen bleach and builder (Wang et al., 1995). Besides, the mentioned specification, the enzyme also needs to be effective at lower concentration and possess broad substrate specificity (Wang et al., 1995). As a role of additives, lipases are required to be active and stable at high temperatures with alkaline pH conditions.

Medical and pharmaceutical products

Microbial lipase is used for the production of pure enantiomers through resolution of racemic mixtures in the pharmaceutical industry. They are also helpful in the production of chiral molecules such as prostaglandins, cephalosporines, nonsteroid, some anti-inflammatory drugs as hydantoins, and penicillinsa. Some chiral molecules are used as herbicides in the agrochemical industry. (Fernandez et al., 2006 a,b). Lipases are used for racemic esters hydrolysis, ransesterification and racemization to yield optically pure enantiomers for the chiral compounds production. Profens (2-aryl propionic acids), an important group of non-steroidal anti-inflammatory drugs that are active only in a specific conformation and are synthesized by the enantioselective esterification catalyzed by lipase from *Candida antartctica* and *Candida rugosa* (Sikora et al., 2014).

Microbial lipase are also used in diagnosis of diseases used as indicators in some health conditions such as acute pancreatitis or injury and diagnosis of fat in heart ailments by determining its activity level in blood serum (Hofmeyr et al., 2014). However, pancreatic lipases are replaced by microbial lipase to treat the cystic fibrosis and pancreatitis and also act as activators to detect tumor necrosis factor which can help in the treatment of malignant tumours (Ye et al., 2011). Recent studies showed that, Angina pectoris, hypertension and other vascular disorders can be effectively treated with diltiazem (a calcium blocker). The production of diltiazem requires pure trans-methoxylphenylglycidic acid methyl ester (MPGM) and the bio-resolution of MPGM was efficiently catalyzed by the lipase from *Serratia marescens* (Pan et al., 2016)

In the perfume and cosmetic industry, lipase is used to produce a number of surfactants and aromatic products and ointments to be used in personal care products.

Biosurfactants bases such as monoacylglycerols and diacylglycerols are produced through transesterification of lipase-catalyzed mixture of racemic intermediates. Further, the esters

of cinnamic acid, wax, ferulic acid and ellagic acid, are synthesized through lipase-catalysis that enhances the flavour and fragrance of compounds. Lipases also act as precursors for some of pharmaceuticals products and as additives in sunscreen and cosmetics products (Ganguly et al., 2015, Kim et al., 2015). Microbial lipases act as primary components in topical creams or orally administered drugs that support in weight loss due to removal of fat (Sonne et al., 2015). An example of the growing patented lipase-catalyzed processes involved in the cosmetic industry by using *P. fluorescens*-and *P. cepacia* derived lipases to produce the menthol esters that provide a peppermint flavor and fragrance in mouth washes and shaving creams. Another example of bacterial derived lipase is resolving racemic rose oxides by using *P.cepacia* to achieve the bromomethoxylation which is a principal constituent in many fragrance products (Sharma & Kanwar 2015).

Degreasing of leather

In leather industry, lipases are used for even colouring and cleaner presence. Microbial lipases help in the production of waterproof leather, which are used in making of leather for car upholstery. Microbial lipases provide two main advantages as fat dispersion and production of waterproof and low-fogging leathers (Choudhary et al., 2004). Alkaline lipases are applied in combination with protease during soaking and liming. Proteases help open the membranes surrounding the fat cell, making the fat accessible to the lipase. The fat becomes more transportable and the breakdown products emulsify the intact fat, which get dispersed throughout the pelt so proper de-greasing with surfactants is not necessary. This simplifies the production of waterproof and low-fogging stock. Microbial lipases can also be applied in the processing of acid as pickled skin or wool and fur, or semiacid for wetblue wool.

Environmental applications

Microbial lipase is used for different environmental purposes such as biodegradation of petroleum hydrocarbons in soils carried out by indigenous microorganisms, which are able to degrade these contaminants by producing lipase etc. Microbial lipase is used in the treatment of triglycerides and waxes from the effluent of pulp and paper industry. The industrial effluent which are rich in oil can be treat by lipase. Microbial lipase is also used in the degradation of organic debris as well as sewage treatment. It is widely used in the biodegradation and bioremediation of toxic substances. Microbial lipase is also used in the production of biodiesel, lubricants, and biokerosene from renewable energy sources by transesterification of vegetable or animal oils. They are also used to produce additives which decreases the viscosity of biodiesel. Some other applications include biopolymer production such as polysaccharides, polyphenols, and polyesters, lipophilization, via lipase, production of biosensors, and phenolic acids and antioxidants modification (Solanki et al., 2016). Microbial lipase is also used in textile industry for washing of cloth and jeans.

Microbial lipases can take the top position in the area of enzyme application in near future. The information reported in the literature shows over 1000 publications per year from numerous fields like production, purification, biochemical catalysis, industrial and commercial applications and medical purposes. Therefore, a great understanding is necessary in all aspects of microbial lipase production including the upstream and downstream processes especially factors which affect the lipase production, stability, activity and reactions (Pandey et al., 1999). In future lipases will dominate the global enzyme market. Therefore, a detailed understanding of lipases production is required using conventional and renewable raw material by using different fermentation techniques. Besides, identification of potential microorganisms and factors involved in the lipase production would be necessary. However, more study is required to get the right microbial

strain through screening and selection, appropriate modification in medium components, and identification of physico-chemical parameters (Salihu and Alam, 2012).

Generally, majority of enzymes used for industrial purpose belong to microbial origin. In 1960, the total sales of industrial enzymes were only a few million dollars annually, since the market has grown enormously (MU et al., 2019). New research is exploring to improve the understanding of the production biochemistry, enzyme recovery methods, fermentation processes, and low-cost enzyme production.

Advances made in the procedure of enzymes application have greatly extended the demand for enzyme. Most of the industrial enzymes such as lipases are hydrolytic in nature. The mechanism of lipase production from microorganisms is generally dependent on cellular regulatory mechanisms provoked by substrate components. Although, there are many different techniques used to enhance the microbial lipases production through the optimization studies, which involve substrate composition, culture condition, and fermentation process (Salihu et al., 2012). Generally, the new approaches are chosen to improve the fermentation process by initially choosing of an effective culture medium for lipase production which include selection of growth factors, carbon and nitrogen source, inorganic salts constituents, and trace elements. Some studies show that agro-residues can give acceptable results in enzyme production, but it is common to select a suitable medium with optimal nutrient concentrations (Ooijkaas et al., 2000). Based on the substrate composition, physical parameters such as pH, temperature, moisture content, size of inoculum, and substrate porosity are some other parameters that are considered to enhance the enzyme production (Rigo et al., 2010). The raw materials used in the solid-state fermentation (SSF) contribute to the total production costs. Thus, substrate cost reduction would be another suitable strategy to increase the production efficiency of any bioprocess techniques (Dominguez et al., 2003).

1.6 Aims and objectives

In the present study, the psychrotolerant and halotolerant bacteria were isolated from temperate region of Leh Ladakh (India) and coastal region of Dwarka, Gujrat (India), respectively. The growth pattern, characteristics and enzyme production of isolated extremophilic strains under extreme environmental conditions has been studied. An additional objective is to observe the isolated bacterial strains and biodegradation capability for its environmental benefit. The intermediate products during biodegradation were screened and identified by modern techniques, such as, HPLC-MS and GC-MS/MS.

The aim of the study is to investigate the lipase producing ability of the isolated bacteria with following specific objectives:

1. **Collection of soil samples and isolation of psychrophilic bacterial isolates.**
2. **Morphological identification and biochemical characterization of the isolated bacteria.**
3. **Screening of the bacterial isolates for production of extracellular enzymes having industrial and environmental applications.**
4. **Study on the factors influencing the bacterial production of enzyme and their structural and functional ability.**
5. **Kinetic study on the enzyme activity and optimization of the factors to enhance the efficacy of enzymes.**

CHAPTER 2

CHAPTER 2
REVIEW OF LITERATURE

2.1 Introduction to lipase

Lipases are known to play numerous physiological functions in eukaryotes. They are key components of lipid and lipoprotein metabolism in eukaryotes (Sharma et al., 2001). The cell density of the microorganism activates the lipase production which is enhanced during the stationary phase or in the beginning of growth phase of microorganism in order to use the stored lipids (Olukoshi and Packter 1994, Wagner and Daum 2005). In most of the microorganisms, lipids and fatty acids are used as carbon sources to induce the extracellular enzyme production, which make them to grow on spoiled soil.

2.2 Mode of catalytic mechanism of lipase

Microbial lipase catalytic mechanism starts by an acylation process. This process consists the transfer of a proton between the aspartate, histidine and serine residues of the lipase enzyme, which causes activation of the hydroxyl group of the catalytic serine. As a consequence, hydroxyl residue of the serine, with subsequent increase in nucleophilicity, attacks the carbonyl group of the substrate. It was observed that the first tetrahedral intermediate is formed with a negative charge on the oxygen of the carbonyl group. The role of oxyanion hole is to stabilize the charge distribution and reduce the state energy of the tetrahedral intermediate by forming at least two hydrogen bonds. The process of deacylation takes place, where a nucleophile attacks the enzyme, releasing the product and regenerating the enzyme. This nucleophile can be either water in the case of hydrolysis or an alcohol in the case of alcoholysis (Bornscherner 2002, Gupta et al., 2004). Lipase catalyzed reactions are classified into three important types:

I. **Hydrolysis:** Enzyme hydrolysis is considered an important process in enzyme technology, which is useful for industry. Specific hydrolases such as lipases, degrade lipids and other esters are used in a variety of scientific and industrial processes (Papamichael et al., 2012). The hydrolysis of natural and artificial esters is a unique reaction type that may be possible due to the opposite polarities of hydrophobic substrates, and hydrophilic catalysts and products. This reaction mostly occurs in aqueous or organic solvent interface, although the interfacial composition is a matter of further research concerning the reaction microenvironment. Since many years ago, works reported on the feedback mechanism of ester hydrolysis, include the digestion of triglycerides, could offer important information regarding the understanding and regulate the reaction course (Aloulou et al., 2006; Reis et al., 2009). Hydrolysis is currently used in the food industry where production of fatty acid, di-glycerides, monoglycerides are needed. It is also used as a flavouring agent for dairy products. This reaction is helpful in the production of detergents for laundry and household purposes.

II. **Esterification:** Enzyme-catalyzed esterification has gained increasing attention for numerous applications, due to the significance of the enzyme derived products. More specifically, the lipase-catalyzed esterification reaction has attracted the researcher's interest during previous years, due to increased use of organic esters in chemical and biotechnological industrial applications (Torres and Castro, 2004). So, esterification technology of lipases was developed (Okumura et al., 1979) and numerous microbial lipases have been engaged in investigation by using either primary or secondary alcohols, or both, free-solvent systems, or organic solvents. The enzyme reaction pH, temperature, mixing rates, and water content are some other important factors, which influence the ester yield (Zaks and Klibanov, 1988).

III. **Transesterification:** It occurs when the acid moiety of an ester is replaced with another one viz. as if the acyl donor is a free acid, the reaction is called acidolysis and if the acyl donar is an ester, the reaction is interesterification. However, if the acyl donor is an ester as in alcoholysis reaction, the nucleophile alcohol acts as an acyl acceptor. (Divakar and Manohar 2007).

2.3 Cold active lipases

Cold-adapted lipase producing microorganisms are functionally effective at low temperatures with high rates of catalysis in comparison to the mesophilic and thermophilic lipase producing microorganisms. The information of cold active lipolytic enzymes is increasing at a speedy and stimulating rate. Cold active lipases are structurally improved due to increased flexibility of the polypeptide chain that enable a flexible binding of substrates at low temperature (Joseph et al., 2007). An attempt has been made by Arpigny et al., (1997) to understand the molecular modelling of lipase from *Pseudomonas immobilis*. The study revealed some specific features of cold-adapted lipases such as low proportion of arginine residues as compared to lysine, reduced content of proline residues, a small hydrophobic core, small number of salt bridges and of aromatic-aromatic interactions.

Psychrophilic microorganisms producing cold adapted lipases may be a good source of polyunsaturated fatty acids required by the pharmaceutical industry. It is because of their exceptional ability for precise regioselective reactions in organic solvents variation with wide range of substrates then enable lipase important biocatalyst in biomedical applications (Margesin et al., 2002). Cold-active lipase are potentially using as additives in detergent industry under low temperature. The advantage of cold active lipase in cold washing is it decreases the energy consumption and wear and tear of textile fibres (Feller and Gerday,

2003). Lipases are stable in detergents containing protease and activated bleach systems. Cold-adapted lipase removes the stains of fatty acid into more hydrophilic substances that are easier to remove compare to non-hydrolyzed stains (Fuji et al., 1986). Stead (1986) and Coenen et al., (1997) reported that cold-active lipase from psychrotrophic *Pseudomonas* sp. and some moulds of *Rhizopus* sp. and *Mucor* sp. played havoc with milk and dairy products and soft fruits juices. Cold active lipase from *Pseudomonas* strain P38 is extensively used in non-aqueous synthesis of n-heptane of the flavouring compound butyl caprylate (Tan et al., 1996).

2.4 Thermostable lipases

Most of the industrial processes in which lipases are used function at a high temperature above 45°C. Generally, microbial lipases stable above 40°C are required for commercial applications (Wang et al., 1995). Thermophilic microorganisms are potential and alternative source of thermostable lipases (Brock 1985). Thus, it is required that industrial lipases must have an optimum temperature around 50°C (Sharma et al., 2002). Several *Bacillus* sp. are the main source of thermostable lipases (Kim et al., 1994; Schmidt et al., 1994). While majority of the microbial lipases are active at pH of 7.0 and at temperature 60°C, but lipases from *B. thermoleovorans* and *R. oryzae* strain are functionally active at extreme pH and high temperature (Dong-Woo et al., 1999; Abel et al., 2000). Among the required characteristics of lipases, alkali tolerance and thermostability are important for industries (Kulkurani and Gadre, 1999). Hence, there is a constant search for extremely active lipolytic enzymes with specific stability towards pH, temperature, ionic strength and organic solvents (Lee and Rhee, 1993). Although, few lipases are reported to show maximum activity at 100°C, but their half-lives are observed to be very short (Rathi et al., 2000).

Chakravorty et al., (2011) investigated a detailed *in silico* analysis of several structural features that contribute to thermal stability of lipases. The result of the analysis revealed that each thermostable lipases adopt to exceptional strategy and display improved stability at high temperatures due to 3D arrangement of amino acids. Some of the strategies include (1) increase in number of titratable amino acids near the active serine residue and (2) increase in the charged residues at surface and decrease in the β-branched residues at helices. *Bacillus* lipases are known to display improved thermostability by growing percentage of Gly residues in loops and poly Ala residues in the lid, but decreasing percentage of free Cys residues, and switching thermolabile residues with those amino acids which show high helix propensity. Thus to fulfil the global requirement of the lipases for industrial procedure, thermostable lipases are highly needed.

2.5 Alkaline stable lipases

Alkaline lipase producing microorganisms have been isolated from water, soil, and some other extreme environments. Alkali tolerant lipases are required in the formulation of detergents because enzyme-based detergents exhibit higher cleaning capability in comparison chemical-based detergents. Alkaline lipases consist some additional characteristics such as organic solvent tolerance and thermal stability make them superior for use in numerous biocatalytic reactions. Some other applications include flavor synthesis and bioremediation in food formulation and conservation. Thus, the metabolic adaptations of microorganisms not only help them to ensure their survival, but also enable them to produce of lipases with required characteristics. These adaptive properties include the maintenance of homeostasis of the cell, which depends on the permeability of some ions as Na^+, K^+ and $H^+$and transport of the buffering capacity of the cytoplasm (Booth I 1985, Evans M et al., 2002), and adjustment of membrane phospholipids. The isomerization of unsaturated fatty acids from cis to trans and the induction of specific stress proteins in some

alkalophilic or alkaline tolerant lipase-producing microorganisms, such as *B. subtilis* is controlled by sigma factor (σ^W) (Lonetto et al., 1994 and Wiegert et al., 2001).

Yoo et al., (2011) reported *Ralstonia* sp. CS274 lipase has an optimum pH between 8.0–9.5. The purified lipase was tolerant to ionic and non-ionic surfactants, organic solvents and metal ions, which helps in its industrial application, especially for non-aqueous biocatalysis. *Staphylococcus aureus* strain PP1 was found as leading strains in soil samples in the groundnut rhizosphere, which was capable of providing an alkali-thermostable lipase with an optimum pH of 8.0. A wide range of stability and activity are reported between pH 5.0-11.0. The maximum stability was reported at pH 9.0, where the enzyme retained its maximum activity after 20 hr of incubation at 37°C. However, pH 8.0, appeared to be the optimum pH for the enzyme activity, as a 90% activity was retained after 20 hr of incubation at 37°C (Sarkar et al., 2012).

2.6 Acidic lipases

Acidophilic microorganisms undergo some adaptive steps to persist in the acidic environment, which include the selective permeability of the membrane to protons, fortification of macromolecular structures, chaperonins production and other acid shock proteins as DnaK, GroEL and HtrA. The alteration in membrane fatty acid saturation controlled by monounsaturated fatty acids and longer chain fatty acids. It is also controlled by the gene that regulates membrane stability, composition and its activity (Cotter et al., 2003, Jayaraman et al., 1997, Quivey et al., 2000). Thus, the acidic lipases producing microorganisms display unique characteristics at low pH.

Aspergillus niger NCIM 1207 strain produced an extremely acid stable lipase which retained its activity at pH 1.5 in solid state fermentation (SSF) containing wheat bran, and olive oil. This lipase showed optimum activity at pH 2.5 and 45°C temperature. The enzyme

retained 63% of its activity after 24 hr of incubation. Interestingly, the lipase also revealed thermal stability by retaining 63 % of activity at 7°C for 5 hr (Mahadik et al., 2002). Colin et al., (2010) have reported that *Aspergillus niger* produced acidic lipase at pH 3.0 in submerged culture using olive oil as the inducer, with potential application in pharmaceutical industry.

Thermostable lipases from *B. stearothermophilus* SB-1 and *B. licheniformis* SB-3 isolated from compost heaps, soils and oil industry effluents are known as acid tolerant and they exhibit high enzyme activity of about 70% and 50% respectively, when incubated in an acidic medium at pH 3.0 at high temperature (50°C) (Bradoo et al., 1999). Ramani et al. (2010) revealed that *P. gessardii* isolated from slaughterhouse waste containing beef tallow produced an acidic lipase with its maximum activity (139 U/ml) at pH 50 and 37°C. Similarly, *Bacillus pumilus* produced an acid tolerant and thermostable lipase with a maximum enzyme activity of 1,100 U/ml at 50°C at pH 1.0, after 96 hr of incubation period. The acidic lipase also exhibited a 96% hydrolytic conversion of palm oil containing waste water after 3 hr at 50°C with pH 1.0 (Saranya et al., 2014a). Acid tolerant lipases have potential application in wastewater treatment, processing of leather and medical applications. In the food industry, the synthesis of aroma and flavour containing compounds are synthesized by acidic lipases (Mhetras et al., 2009).

2.7 Organic solvent tolerant lipases

Lipases used in most of the industrial chemical reactions like esterification, transesterification, racemic resolution, and biodiesel production are required to retain their activity in the presence of organic solvents (Jaeger and Eggert, 2002). Organic solvents usually inactivate lipase activity by reducing conformational flexibility, exposing hydrophobic residues, stripping of crucial water, and interfacial inactivation, etc. Though,

lipases are interfacial enzymes which retain their stability better than other enzymes in the presence of organic solvents (Doukyu and Ogino, 2010). Hence, there are several reports on organic solvent-tolerant lipases from many bacteria such as *P. aeruginosa*, *Pseudomonas* sp., *B. sphaericus*, *B. megaterium*, *B. thermoleovorans*, *Staphylococcus saprophyticus*, *Burkholderia cepacia*, and among fungi, *Penicillium chrysogenum*, *R. oryzae*, and *Cryptococcus* sp (Javed et al., 2018).

Bose and Keharia (2013) reported that lipase produced from *Pseudomoans aeruginosa* AAU2 is stable in organic solvent and retains more than 70% of its activity after 24 hr of incubation. Lipase catalysed reaction in non-aqueous medium generally prefer organic solvents with a high log P value due to their less effect on stripping of the water from lipase molecules. It has been observed that lipase activity could be enhanced in the presence of high concentration of iso-octane (75% v/v) in *P. aeruginosa* AAU2. It was suggested that increase in the lipase activity was due to some conformational changes associated with the interaction of amino acid residues, specifically hydrophobic ones present in the lid that covers the active site of enzyme that causes enzyme to remain in an open conformation (Rua et al., 1993). It was reported that lipase from *P. fluorescens* P21 was found to be stable after 2hr of incubation in non-polar solvents such as heptane, hexane and styrene, with a residual activity of 91.4%, 94.1% and 83.5%, respectively (Cadirci et al., 2010). Yang et al., (2007) reported that recombinant lipase from *Burkholderia cepacia* strain G63 showed maximum lipase activity (863 U/ml) after 48 hr and was stable in the presence of 50% (v/v) methanol, ethanol, tert-butyl glycerol and alcohol. The recombinant lipase after 72 hr hydrolysed 88% soybean oil to fatty acids to be used for biodiesel production. Similar findings was reported by Ebrahimpour et al. (2011) in *Geobacillus* sp. strain ARM recombinant lipase which showed 1623-fold higher enzyme activity than wild-type strain. Further study revealed that lipase is a thermostable, organic solvent tolerant and 1,3-

regiospecific with respect to triolein. These studies highlighted the importance of organic solvent-tolerant property of lipases for numerous industrial applications. Nasaruddin et al., (2014) reported that methanol tolerant lipase from yeast *Rhodotorula mucilagenosa* P11I89 used as acyl acceptor in esterification and transesterification reactions, but due to inhibitory nature of methanol towards lipases, it was used for production of fatty acid methyl ester (FAME) using palm oil and methanol (1:3, 1:4, 1:5, 1:6) at different mass ratio. It was observed that a higher molar ratio of methanol supported higher FAME yields (Srimhan et al., 2011).

2.8 Salt tolerant lipase

Halophilic or halotolerant bacteria are found to exhibit under high salt tolerance and are present in saline environment or salterns where seawater or brine is stored and permitted to evaporate for salt harvesting. The microorganisms requiring 1-10% of NaCl for their growth are kept in the category of moderately halophilic (Ghasemi et al., 2011). Larsen (1986) catagorised halophilic microorganism under four category according to their salt tolerance limit. Those microorganisms require ≤1% NaCl for their growth and metabolism and are considered non-halophilic, whereas those growing in the presence of 2–3 % and 5–10 % NaCl are termed as slightly halophilic and moderately halophilic microorganisms respectively. Microorganisms requiring more than 10 % NaCl are termed as extremely halophilic. There are two main strategies used by halophilic microorganisms to survive at high salt environment (Oren 2002; Karan et al., 2012). Microorganisms using the first strategy by actively impel. The salt out of the cell, while importing solutes that are more friendly with its intracellular contents such as ectoine and glycine betaine (Lentzen and Schwarz 2006; Detkova and Boltyanskaya 2007; Siglioccolo et al., 2011). These halophilic microorganisms generally do not exhibit wide adaptive changes in intracellular enzymes, but their cell walls, transporters, and periplasmic proteins are

typically modified to function in high salt environments. The halophilic enzymes have many acidic amino acid residues and a reduced number of hydrophobic residues on exteriors of proteins to prevent protein aggregation and electrostatic interaction (Rao and Argos 1981; Bolhuis and Thomas 2008).

Balaji et al. (2014) reported that *Bacillus* sp. VITL8 isolated from a hydrocarbon-contaminated site retained approximately 55 % of its enzyme activity at pH 10.0 under 60°C temperature with 3 M NaCl concentration. The lipase produce by this bacteria was stable in different organic solvents and showed enhancement in the activity by 25 % and 20 % in the presence of 25 % (v/v) of acetonitrile and butanol, respectively. The hydrolytic specificity of the lipase was found towards olive oil and sunflower oil (long chain fatty acids). Thus, the speciality of halotolerant lipase produced by *Bacillus* sp. VITL8 could be applied in several industrial processes as a green technology. Li and Yu (2014) revealed that a salt tolerant strain of the genus *Haloarcula* G41, isolated from soil samples of Yuncheng Salt Lake showed lipolytic activity in the culture media containing 20 % NaCl or 15 % Na_2SO_4 with optimum activity at 70°C (pH 8.0) with 15 % NaCl. Some additional properties of *Haloarcula* sp. lipase was transesterification of soybean oil with methanol in the presence of tert-butanol, which enhanced the biodiesel yield by more than 80%.

Ozcan et al., (2009) studied 18 archaeal strains isolated from different halophilic environments in Turkey. These strains were screened for lipolytic enzyme production. The screening result revealed that 5 out of the 18 isolates, coded as A43, B53, E7, A138 and B49, showed higher lipase activities in the presence of NaCl up to 4 M, with an optimum temperature of 60–65°C and pH 8–8.5.

Perez et al., (2011) reported that *Marinobacter lipolyticus* SM19 produced lipase with potential application in the enrichment and synthesis of unsaturated fatty acids. It was found that lipase could enhance the eicosapentaenoic acid content of fish oil by approximately 45

%. Some other properties include the stability of lipase activity at 80°C on acyl substrates with short-to-medium chain length and thus, it was found to be thermostable in various organic solvents.

2.9 Applications of microbial lipase

Microbial lipases constitute an important group of biotechnologically valuable enzyme, used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics, and pharmaceuticals due to their versatility, (Rubin and Dennis, 1997a,b; Kazlauskas and Bornscheuer, 1998). Lipases are considered as versatile biocatalysts because of their hydrolytic activity on triglycerides as well as their capability to catalyze other reactions such as esterification, interesterification, acidolysis, alcoholysis, and aminolysis. As hydrolases, lipases do not require cofactors. Most regioselective lipases are known to act preferentially on ester bonds at the *sn*-1 and *sn*-3 position of the triglyceride structure, whereas few lipases are active at the sn-2 position (Houde et al., 2004). Microbial lipases are found to retain their activity over a wide range of temperatures. Some microbial lipases exhibiting higher activity are commercialized for specific applications, as while others can be used in different industrial fields. However, number of commercial lipases are higher in number then the industrial lipase. The lipase mediated applications remain limited owing to their high cost. The low number of available lipases in industry is due to low performance of lipase-mediated processes. Nevertheless, lipases presently being used in the food, detergent, and pharmaceutical industries (Houde et al., 2004) can be also used to accelerate the degradation of fatty waste (Masse et al., 2001) and polyurethane (Takamoto et al., 2001). The high selectivity and mild conditions associated with lipase-mediated transformations have made them very attractive for the synthesis of a wide range of natural products, pharmaceuticals, fine chemicals, food ingredients (Schreier,

1997) and bio-lubricants (Dörmö et al., 2004). The renewed interest in this enzyme is primarily due to of their role in pathogenesis and their increasing use in biotechnological applications

Bacterial lipases are more preferred than other microbial lipases because of their physiological and physical properties.

- A large amount of purified lipase can be made available, i.e. ease of mass production.
- Bacterial lipases are generally more stable than animal or plant lipases.
- Bacterial lipases are active under ambient conditions and the energy expenditure required for reactions at elevated temperatures and pressure is eliminated, which reduces the destruction of labile reactants and products.
- Thermophilic bacteria and their enzymes are stable at high temperatures and remain functional under harsh chemical environments, which is added advantage in industrial uses.
- Due to specificity of enzymes, unwanted side products that normally appear in the waste stream are reduced or eliminated.
- The use of enzymes can decrease the side reactions and postreaction separation problems.
- Lipase catalysed processes are cost-effective in comparison to the traditional downstream processing.
- Lipases remain active in organic solvents during their industrial applicability.
- When immobilized lipases are used under typical ‘industrial’ conditions, reactor temperatures can be high as 70°C and may be for longer periods.

Microbial lipases belong to the family of hydrolases that act on carboxylic ester bonds. The hydrolytic properties of lipase include breaking of triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. Lipases versatility makes them a better choice for potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries (Houde et al., 2004).

In the production of cocoa butter equivalent to human milk fat substitute "Betapol", microbial lipase plays an important role by catalysing transesterification in organic solvents which makes them potent for emerging industrial application such as production of pharmaceutically important polyunsaturated fatty acids (PUFA) rich/low calorie lipids, "designers fats or structured lipid" and production of biodiesel from vegetable oils (Nakajima et al., 2000, Jaeger et al., 1998).

In the manufacturing of butter and margarine, lipase is useful in the preparation of glyceride by interesterification and hydrogenation techniques. The conventional method for interesterification reaction involves the presence of a catalyst such as sodium, sodium methylate. However, the conventional method is not selective with respect to esterification of a fatty acid at a reactive position with glycerine. On the other hand, lipase act as a catalyst in the interesterification process, where this process requires the presence of water to activate the lipase. Water plays an important role by causing hydrolysis of interesterified glycerides with reduction in yield of the glyceride product. Therefore, there is need to improve the yield of glyceride products by an interesterification reaction (Tanaka et al., 1981).

The lipase modified lipids into fats or oils, modelled to be healthier with some specific functional and physical properties, are more suitable for food industry. These modifications consist changes in fatty acid content, changes in the position of the fatty acid on the glycerol

backbone and changes in the length of the fatty acid as well as its degree of saturation. Use of structured lipids may be the most effective approach to include the desired fatty acid in the diet for nutritive and therapeutic purposes. Lipid possessing higher value-added attributes can also be produced through lipase-catalyzed interesterification reactions and/or by acidolysis of a less desirable and cheaper lipid. (Houde et al., 2004).

Microbial lipases used in the industrial hydrolysis of tallow offers a large number of advantages because of less heat requirement (about 50°C) to melt the tallow. Due to low temperature requirement there is less degradation of unsaturated fatty acids and therefore, natural fatty acids can be easily gained without distillation, even from highly unsaturated oils. Because of their nutritional value, undegraded poly-unsaturated fatty acids may be important to preserve in the production of food additives such as mono- and di-glycerides. Therefore, depending on the specificity of the lipase and the raw material, partial hydrolysis could yield a concentrated or purified mixture of fatty acids and/or partial glycerides with some exclusive properties, which are not found in bulk fatty acids obtained from the complete hydrolysis of tallow. Diacylglycerols are the main components of new cooking oils (ex: Econa, Kao corp.). These cooking oils contribute to reduce the blood triglycerides which helps in preventing the accumulation of body fat and high blood cholesterol levels.

2.10 Isolation and screening of lipase producing microorganisms

There is a high demand for isolation of new microorganisms, which are sources of lipases with different catalytic characteristics. It has been reported that lipase-producing bacteria are found in different types of waste such as industrial wastes, waste from vegetable oil

processing factories, dairy plants, and soil contaminated with oil and oilseeds, among others (Sharma et al., 2001). The environment surrounded by oil mill effluent may be a good source of lipase producing microorganism (Sztajer et al., 1998; Mobarak-Qamsari et al.,

2011). Thus, the exploration of new habitat of microorganism offers the possibility of a new enzyme. Furthermore, the newly isolated lipase producing microorganisms can reveal new scientific facts and commercial applications (Ko et al., 2005).

Sirisha et al. (2010) isolated a bacterial strain from oil and fat contaminated soil of dairy and oil refinery. The isolated bacterial strain was identified as *Staphylococus* sp. and coded as L2. It was screened for extracellular lipase production on tributyrin agar medium. The maximum lipase production was found to be 25 U/ml after 48 hr of incubation. Lipase production was enhanced in the presence of palm oil as carbon source and peptone as nitrogen source at 36℃ at pH 7.0.

Bharathi et al. (2019) isolated eight bacterial strains from petrol spilled soil by serial dilution technique. All the strains were screened on the basis for extracellular lipase production. Five of them were identified as lipase producing strains when grown on tributyrin agar medium with olive oil as an additional carbon source. The maximum production of lipase was observed in the presence of 3% sucrose and 5% yeast extract at 37 °C with pH 6.0 ± 0.5 under submerged fermentation condition. The lipolytic stains were identified on the basis of their morphological and biochemical characterization as *Corynebacterium* sp., *Streptococcus* sp.,*Escherichia* sp.,*Proteus* sp., *Bacillus* sp., *Staphyl ococcus* sp., *Pseudomonas* sp., and *Klebsiella* sp.

Pathak et al. (2015) isolated three thermostable bacterial strains from vicinity of local oil extraction industry, dairy industry and pulp paper industry from Maharashtra. The isolated strains were coded as TS1, TS2 and TS3. Based on their screening on tributyrin agar medium TS1 was the most efficient lipase producing strain. The titrimetric assay method showed that TS1 produced 66 U/ml thermostable lipase at pH 7.0 and temperature 50°C. Vasiee et al. (2016) isolated a lipolytic strain from rice flour and screened for lipase

production by Rhodamine B agar plate method. The strain was identified as *Bacillus cereus* based on 16S rRNA gene sequencing. Optimization of maximum lipase production was performed by using Plackett-Burman design (PBD) and response surface methodology (RSM). The maximum lipase activity in the isolated strain was observed as 177.3 ± 20 U/ml under modified media. Lipase production was found to be 1.83-fold higher as compared to predicted value (324 U/ml).

Sharma et al. (2017) explored a lipase producing bacteria from oil contaminated soil of service station at Himachal Pradesh. The isolated strain was identified as *Bacillus methylotrophicus* PS3 based on 16S rRNA sequencing method. It was screened for hydrolysis of lipid by adding 1 % tributyrin in culture media. The lipolytic activity was determined by titrimetric method with the help of olive oil. After optimization the isolated strain produce 320 IU (International Unit) at 40°C with 1 % tributyrin and pH 7.0.

Rabbani et al. (2015) isolated 64 bacterial strains from different soil samples. Among them only four strains showed the lipolytic activity in the presence of Rhodamine B in Horikoshi media. The screened strains produced coloured halo on the Rhodamine B culture plates, which confirmed the production of lipase enzyme. Morphological and biochemical characterization of the strain showed that the isolated strain was *Bacillus subtilis*, a Gram-positive rod.

Khosla et al. (2017) isolated a lipase producing pyschrotolerant bacterial strain from Pangong Lake. The modified culture medium containing phenol red indicator and 1% tributyrin as additional carbon source was used to identify the lipolytic strains. The utilization of tributyrin by isolated bacterial strains was considered as an indication of lipase activity. The strain was identified as *Pseudomonas* sp. ISTPL3 based on 16S rRNA gene sequencing.

Kumar et al. (2012) isolated a lipolytic bacterial strain from common garbage site of Karnal city, Haryana, India. The bacteria were isolated by serial dilution on modified nutrient agar plate containing tributyrin, rhodamine olive oil, tween 20 and tween 80 agar plates. The zone of hydrolysis present on TBA plate confirmed the production of lipase enzyme. The isolated strain was identified as *Bacillus* sp. DVL2. Sagar et al. (2013) isolated 18 bacterial strains from the domestic dumping site of Tezpur, Assam. The isolates were screened for lipase production on the tributyrin agar plate media. Two of them coded as TU-L1 and TU-L2 produce a large size (23 mm and 21 mm) halos on the TBA plates. Morphological and biochemical analysis revealed that the isolated strains belong to genus *Staphylococcus*.

Mehta et al. (2012) isolated 32 bacterial strains from a soil sample of hot spring. One of them showed maximum lipase production in the minimal salt medium containing olive as a substrate. The screened bacterial strain was coded as TS-4 which was identified as *Geobacillus* sp.

Hasan-Beikdashti et al. (2012) isolated lipase producing bacterium *Stenotrophomonas maltophilia* from oil-contaminated soil samples from various locations in Tehran, Iran. The screening was done by adding Rhodamine B (0.001%, w/v) and olive oil (3%, v/v) in nutrient medium. The production of lipase enzyme was confirmed by shiny orange halos when observed under ultraviolet light (350 nm).

Sivaramakrishnan et al. (2016) isolated a bacterial strain from the soil sample of metallurgical industry. An enriched medium containing olive oil as additional substrate as was for isolation of lipolytic bacterial strains. The screening of lipase producing bacteria was done by rhodamine B-olive oil agar plate methods. The isolated strain was identified as *Bacillus* sp. based on 16S r RNA gene sequencing method. The microorganisms producing lipase include bacteria, fungi, yeasts and actinomyces.

Sierra (1957) described a simple plate test method for identification of lipase production in microorganisms. This method is based on the hydrolysis of Tween 80 in a solid medium. The lipolytic activity of microorganism produces an opaque zone around the colonies, which indicates the lipase production by particular microorganism. After that, there were some modifications suggested for this primary screening method such as use of different types of Tween surfactants in combination with Nile blue and Cu^{+2} salts.

Kumar et al. (2012) described various substrates for detection of lipolytic activity of microorganism. The tributyrin agar (TBA) is frequently use for the screening of lipase producing microorganism and clear halos around the colonies confirm the production of lipase. Lipase production can also be detected by chromogenic substance as Rhodamine B agar method. Kumar et al. (2012) used Rhodamine B plates to screen lipolytic activity in the *Bacillus* sp. strain DVL2. Hou (1992) reported the modified Rhodamine B plate method for screening of lipase producing bacteria.

Lipolytic activity of microorganisms are generally test on solid media. The agar containing methods can be divided into two categories based on their screening indicators.

- Method based on the substrate hydrolysis of the type of substrate
- Method based on the chromogenic dye used for the detection of hydrolysis zone around colonies (Thomson et al., 1999).

Both methods include a direct contact between the substrate and enzyme. Thus, the content of agar in screening plate media can be reduced to increase diffusibility of microbial extracellular enzyme (Hou and Johnston, 1992). The first method of screening depends on the lipase substrate used to visualize the microbial colonies on growth media. The screening media was prepared by adding tributyrin or Tween 20/80. After the incubation period clear or opaque halos formation around colonies confirmed hydrolysis of substrate. This

screening method have several benefits as it does not require specific dye which may prevent the inhibition of microbial growth due to their sensitivity toward dyes. (Thomson et al., 1999).

The other method includes addition of some specific dyes as spirit blue, phenol red or nile blue sulphate to detect the lipase production. These dyes can be used directly to the screening media or growth plates after incubation of microorganism. In the dye-based detection method, pH is responsible for the change in the dye colour. The dye colour changes due to lipolysis of substrate that release fatty acids from triglycerols, the screening may be performed by the combination of substrate such as tributyrin, tween and dyes like nile blue and Victoria blue (Thomson et al., 1999). However, these substrates are not useful for the detection of true lipase because they are also hydrolysed by esterase.

Jones and Richards, (1952) reported that confirmation of lipolytic activity was confirmed by formation of clear halos by using tributyrin as substrate but in the presence of Victoria blue B. The lipolytic colonies were surrounded by dark zones on opaque, light blue background. Microbial lipase can be detected by using fluorogenic dye Rhodamine B that acts as indicator in the presence of olive oil. Rhodamibe B and olive oil added in the growth plates, which become pink due to Rhodamine B. The lipase producing microorganism forms orange fluorescent halos around the colonies under UV light. (Kumar et al., 2012). This orange fluorescent compound is formed due to the formation of Rhodamine B –long chain fatty acid conjugates (Jaeger et al., 1999). The Rhodamine B plates test method is not affected by pH changes and it does not inhibit the growth of microorganism or altered the physiological properties (Thomson et al., 1999).

2.11 Production of microbial lipase

Microbial lipases are mostly extracellular and their production is greatly influenced by medium composition besides physicochemical factors such as pH, temperature, carbon

source, nitrogen source, inoculum size or metal ions (Treichel et al., 2010). Microbial lipase is generally produced in submerged culture media (Ito et al., 2001) but solid-state fermentation method is also used for production (Singh et al., 2010). The effect of cultivation conditions on lipase producing microorganisms were observed by subjecting them to shaking flask experiments under different environmental conditions (Yang et al., 2005). The maximum lipase production is dependent on the culture condition and nutritional requirement of microorganisms in submerged culture media.

I. Submerged fermentation system

Microbial lipase is produced by submerged fermentation system (SmF). It is a technique used to cultivate the microorganism in liquid broth medium with exogenous supply of nutrients (Costa et al., 2017). Submerged fermentation system is also called liquid fermentation system. The process of recovery and purification of lipase is easy and better in SmF system rather than others. It is mostly used in the production of bacterial lipase. Some other carbon sources such as olive oil, coconut oil and vegetable oil are commonly used in SmF system. Various oils especially olive oil, palm oil, sunflower oil and almond oil exhibit enhanced yield of lipase production from microbial strains (Coradi et al., 2013; Thakur et al., 2012).

II. Solid state fermentation system

Solid state fermentation system is also called solid substrate fermentation (SSF) system in which microorganisms are grown on solid substrate media to produce the enzyme. This technique is used in food and pharmaceutical industries to produce metabolites from microorganisms (Mussatto et al., 2012; Barrios-González et al., 2012). Mostly, solid substrate fermentation is used for metabolites production from yeast, fungal and mycelial lipases by using several solid substrates. For example, mustard oil was used as oil substrate

for the production of lipase from *Aspergillus* sp. in both SSF and liquid fermentation and the best production efficiency was shown in SSF system (Sethi et al., 2016). The cost of lipase production by SSF is much cheaper than other systems. The economic advantages of lipase production by SSF have been highlighted due to reduced cost of production. It is found that SSF is an economical substitute for large scale production of enzymes by fungi. Therefore, production of lipases by SSF is a worthy and preferred selection than submerged fermentation (Colla et al., 2015).

2.12 Factors influencing microbial lipase production

Several environmental and physico-chemical factors strongly influence the production of microbial lipase. The role of temperature, pH, metal ions and inducers are the most important factors.

2.12.1 Effect of pH on lipase production

The pH of growth medium influences the rate of production of bacterial lipases. Generally, microbial lipases have alkaline pH optima or neutral pH. Some studies suggested that neutral and alkaline pH conditions enhance the production of extracellular lipase in bacterial and yeast cells (Ramakrishnan et al., 2016; Bharathi et al., 2019). Veerapagu et al. (2013) reported that *Pseudomonas gessardii* isolated from oil-spilled soil had a maximum lipase production at pH of 7.0. Sirisha et al. (2010) reported similar findings in *Staphylococcus*. Microbial lipases possess stability over a wide range of pH from 4.0-11.0 (Patil et al., 2011). Taskin et al. (2016) found that *Rhodotorula glutinis* HL25 produced a good yield of when pH of the production medium was maintained at neutral pH. Turati et al. (2019) observed good yield of fungal lipases while maintain the lipase production media under acidic condition (pH 4.0). Kim et al. (2000) studied the effect of pH on the growth and lipase production by *Geobacillus stearothermophilus* KGSHW-1. Lipolytic

productivity was found to be maximum at pH 10.0, the 1477.9 U/mL of lipase yield. The lipase production was found decreased below the optimum value of pH 10.0. Therefore, the result suggested that the bacteria needs neutral or alkaline pH values for maximum enzyme production. The bacterial growth was completely inhibited outside this pH range which revealed the important of pH variation in the growth and cultivation of *Geobacillus stearothermophilus* strain. The effect of acidic pH on the production of lipase was reported by Ramani et al. (2010). They suggested that *Pseudomonas gessardii* produced 156 U/mL of lipase at pH 3.5, using slaughterhouse wastes at 0.31 g substrate concentration. Agobo et al. (2017) reported that *Bacillus subtilis* KPL13 showed a good yield of lipase production at pH 6.0. Priya and Reddy (2015) reported that most of *Bacillus* sp. exhibit optimum pH 7.0 for maximum lipase production.

2.12.2 Effect of temperature on lipase production

Most of the lipase producing microorganism are mesophilic in nature i.e. grow in moderate range of temperature between 25 and 40°C. However, some psychrophilic and thermophilic organisms are found to exhibit optimum production at low and high temperature, respectively. Temperature plays a significant role in the production of microbial lipases. Optimum temperature is necessary for secretion of enzyme in culture medium flask. Bharathi et al. (2019) reported higher biomass concentration of lipase producing bacteria at temperature 37°C isolated from petrol spilled soil. Joseph et al. (2007) reported about some lipase producing psychrophilic strains are *Achromobacter lipolyticum, Acinetobacter* sp., *Bacillus sphaericus*, *Aeromonas hydrophila*, *Pseudomonas fluorescens Morexella* sp., , *Pseudomonas fragi, Psychrobacter okhotskensis,* and *Staphylococcus epidermidis*.

Cherif et al. (2011) reported that an increase in the temperature led to negative effect on biomass production and enzyme activity. The reason for decrease in lipase activity due to rising temperature was found to be associated to either inactivation of enzyme or decrease

in cell growth. Kim et al. (2000), reported the optimum temperature for growth of *Bacillus stearothermophilus* was as 55 ºC, but the enzyme activity was minimum at this temperature. Sifour et al. (2010) suggested that *B. stearothermophilus* produced lipase in a high yield at 55 and 60ºC. Sivaramakrishnan and Incharoensakdi (2016) reported solvent tolerant lipase producing *Bacillus* sp., showed optimum temperature of 37°C for the lipase activity.

Priya and Reddy, (2015) reported that optimum temperature was found 36°C for the maximum lipase activity in *Bacillus* sp. Kulkarni and Gadre (2002) reported a similar finding in the *P. fluorescence* NS2W. Veerapagu et al. (2013) reported that *Pseudomonas gessardii*, isolated from oil spilled soil, showed maximum lipase production at 37°C. Khemika et al. (2012) reported similar findings in *Pseudomonas xinjiangensis* and *Staphylococcus* (Sirisha et al., 2010).

Table 2.1 Optimum pH and temperature of some bacterial lipases

BACTERIAL STRAIN	pH	TEMP (°C)	REFERENCE
Burkholderia ubonensis	8.5	65	Yang et al., 2016
Acinetobacter sp. CR9	8.0	40	Kasana et al., 2008
Enterococcus faecium	10.8	45	Ramakrishnan et al., 2016
Streptomyces lividans	8.0	50	Wang et al., 2016
Acinetobacter sp. RAG-1	9.0	62	Snellman and Colwell, 2002
Pseudomonas aeruginosa	8.0	45	Unni et al., 2016
Bacillus sp.	6.0	30	Etugrul et al., 2007
Bacillus stratosphericus	9.0	35	Gricajeva et al., 2016
Burkholderia multivorans	7.0	37	Gupta et al., 2007
Bacillus amyloliquefaciens	10.0	40	Saengsanga et al., 2016
Bacillus pumilus	8.0	45	Laachari et al., 2015
Fervidobacterium changbaicum Lip1	7.8	78	Cai et al., 2010
Geobacillus thermodenitrificans	9.0	65	Christopher et al., 2015
P. aeruginosa MTCC 10055	9.0	35	Bisht et al., 2012
Pseudomonas gessardii	5.0	30	Ramani et al., 2010
Bacillus pumilus RK31	6.0	60	Kumar et al., 2012
P. fragi NRRL-B727	7.0	30	Sathyavrathan and Jaya 2013
Colwellia psychrerythraea 34H	7.0	25	Do et al., 2013

P. xinjiangensis CFS14	8.0	37	Lomthaison et al., 2012
Acinetobacter baylyi	8.0	60	Uttatree et al., 2010
Halobacillus sp. strain LY5	10.0	50	Xin et al., 2012
Chromohalobacter sp. LY7-8	9.0	60	Li and Yu 2012
Janibacter sp. R02	8-9	80	Castilla et al., 2017
Chryseobacterium polytrichastri	7-8	20	Kumar et al., 2020
Pseudomonas helmanticensis HS6	7.0	50	Phukon et al., 2020
Burkholderia pyrrocinia B1213	8.0	50	Li et al., 2018
Pseudomonas sp. LSK25	8.0	10	Salwoom et al., 2019
B.thermoamylovorans BHK67	7.5	55	Sharma et al., 2018
Pseudomonas aeuriginosa	9.0	30	Ilesanmi et al., 2020
Staphylococcus caprae NCU S6	9.0	40	Zhao et al., 2021
Pseudomonas reinekei	5-9	40	Priyanka et al., 2019

2.12.3 Effect of inoculum size on lipase production

Elibol et al. (1995) suggested that nature and size of inoculum may affect the microbial growth and play an important role in the enzyme production. Lee et al. (1999) reported maximum lipase production when 1% of *Bacillus thermoleovorans* ID-1 inoculum was used. The nutrient and oxygen level present in culture medium supported maximum growth and enzyme production in bacteria, when appropriate size of inoculum was used. Baharum et al. (2003) reported that higher inoculum size may cause loss of nutrient supply and total dissolved oxygen, which may affect the production of biomass and enzyme. On the other hand, low inoculum size may cause reduced production of biomass which ultimately leads to reduced enzyme secretion. It was concluded that lipase production by *Pseudomonas* sp. strain S5 was sharply decreased especially when 10% (v/v) inoculum size was applied. Giuseppin (1984) also proved that the rate of lipase production rate was higher at low inoculum size and relatively high oxygen concentration.

2.12.4 Effect of carbon sources on lipase production

Carbon source are essentially important for the energy production and serves as inducer for

obtaining a high yield of lipid production in microorganism. Ghosh et al. (1996) reported that carbon source requirement for lipolytic activity varies with the microorganism. *Rhizopus nigricans* culture media supplemented with glucose along with triglycerides showed enhanced production of the lipase. Lima et al. (2003) reported that *Penicillium aurantiogriseum* needed an inducer along with carbon source, because when glucose alone utilized as a sole carbon source without inducing the enzyme activity. Hence, they proved the presence of inducer is necessary for lipase production. Microbes produce lipase with induction of lipase producing gene. The use of oil carbon sources such as olive oil, palm oil, and other vegetable oils serves as inducer for lipases production. While, olive oil is known to play a great role in the induction of lipase enzyme in all type of microbial lipase. It has been observed that when 1% olive oil is used in culture medium, the lipase production is induced in *Bacillus* sp. However, very low enzyme activity was seen in the absence of olive oil even after long time cultivation (Sugihara et al., 1991). Papaparaskevas et al. (1992) reported that fructose and palm oil were the best carbon and lipid source for the production of an extracellular lipase by *Rhodotorula glutinis*. While comparing both the fructose and palm oil carbon source, palm oil at a concentration of 2% exhibited about 12-fold more lipase than the fructose medium. Sethi et al. (2016) reported that a good yield of lipase was obtained when mustard seed was used in combination with olive oil cake- sugar cane bagasse in *Aspergillus terreus*. Zarevúcka et al. (2012) suggested that olive oil is best for the production of lipase rather than other carbon source in bacterial strains.

Li et al. (2001) reported that Tween 80 enhanced the production of lipase and also aided better recovery of enzyme produced by *Acinetobacter* sp. along with other vegetable oils widely used for better yield of higher lipases due to their cheap and relative availability (Messias et al., 2009).

Bhushan et al. (2011) used different types of growth medium (M1 to M5) with wide range

and amount of carbon, nitrogen and nutrients to optimize the biomass and enzyme production in *Arthrobacter* sp. (RRLJ-1/95). It was observed that in the culture medium M5 containing 20g/L sucrose and other nutrients produces a high yield of cell biomass at 10 g/L and lipase 1800 U/g. Lin et al. (2006) reported that culture medium containing sucrose showed better lipase yield in *Antrodia cinnamomea* as compared to other carbon source as glucose, galactose, fructose and xylose. Bisht et al. (2012) used different carbon sources such as mannitol, lactose, sorbitol, glucose, fructose and starch to optimize the alkaline lipase produced by *P. aeruginosa* MTCC 10,055. It was found that maximum lipase was produced maximally as a carbon source with starch (960.8 U/mL), followed by glucose (550.9 U/mL) and lactose (476.1U/mL). However, mannitol, sucrose and fructose showed negative effects on lipase production with less lipase secretion. Similar result was reported by Pogaku et al. (2010) and Rathi et al. (2001) who studied the presence of oil and glucose as additional carbon sources for the production of lipase in *Staphylococcus* sp. Lp12 and *Burkholderia cepacia* respectively.

Various types of reports are available related to effect of carbon sources on lipase production. Among the different carbohydrates, it was found that sucrose resulted in maximum lipase production (Fadiloglu et al., 1999). It was reported that *Candida rugosa* showed maximum (5.58 U/mL) lipase activity when culture medium contains yeast extract, peptone, peptone and olive oil but minimum lipase activity (2.81 U/mL) was observed when tryptone and lactose were used in the culture medium. Espinosa et al. (1990) reported that the lipase production was about 1.6 times higher in *Rhizopus delemar* with dextrin as compared to glucose. Few studies suggested that the presence of glucose in the culture medium depressed the lipase production in the presence of olive oil. While, it was observed that when basal medium supplemented with glucose was inhibitory to lipase production which could be due to catabolic repression. Similar result was observed for other lipolytic

microorganisms in which a high level of glucose concentration inhibits lipase production (Lotrakul and Dharmsthiti, 1998, Chen et al., 1992; Mobarak-Qamsari, et al., 2011). Christianah et al. (2012) studied that *Trichoderma virens* and *Hypocrea patella* lipase producing fungi isolated from oil-spilled soil showed maximum lipase production in the presence of sucrose and xylose as these nutrients were utilized for growth and lipase production.

2.12.5 Effect of nitrogen sources on lipase production

Nitrogen is an essential source for the growth of microorganisms and production of microbial lipase. Nitrogen source can be used in various forms as organic and inorganic. It has been observed that various thermophilic *Bacillus* sp. and *Pseudomonas* sp. showed higher yield of lipase in the presence of organic nitrogen source such as peptone and yeast extract (Sugihara et al., 1991; Sharma et al., 2002). Some previous studies suggested that salts, sodium nitrate, peptone, yeast extract, urea and tryptone enhanced the lipase production in all types of microorganisms (Oliveira et al., 2016; Bose and Keharia, 2013; Priyanka et al., 2019; Das et al., 2016). Rodriguez et al. (2006) reported that addition of urea into the culture medium of *Rhizopus* sp. could enhanced the production of lipase. Colonia et al. (2019) reported that peptone in combination with other nitrogen source could increase the lipolytic activity of *Aspergillus* sp. *P. aeruginosa* KM110 showed maximum lipase production in the presence of peptone which suggested that the peptone was the most suitable nitrogen source (Mobarak-Qamsari et al., 2011).

A study on alkaline lipase production from *P. aeruginosa* MTCC 10055 showed that yeast extract could enhance the production of lipase if used as an additional nitrogen source in the cultivation medium. Among the other inorganic nitrogen sources, ammonium sulphate was found to enhance the lipolytic activity in *P. aeruginosa* MTCC 10055, whereas ammonium di-hydrogen orthophosphate inhibited the lipase production (Bisht et al., 2012).

Inorganic nitrogen sources such as ammonium chloride and ammonium dihydrogen phosphate have been also reported to be more effective in some microbes for lipase production (Dong et al., 1999; Rathi et al., 2001; Mobarak-Qamsari et al., 2011). Lima et al. (2003) also reported that ammonium salt was necessary for growth of *Pseudomoans aurantiogriseum.*

It has been reported that with the 0.1% (w/v) and 0.5% (w/v) yeast extract, the lipase production was maximum in *Bacillus* strain A30-1 (Wang et al., 1995) and *Pseudomoans citrinum* (Pimentel et al., 1994), respectively. Yeast extract not only acts as a nitrogen source but also provides vitamins and trace metals in the culture medium that may affect the microbial growth and enzyme production (Gupta et al., 2007).

Bayoumi et al. (2012) reported the effect of various inorganic and organic nitrogen sources on lipase production by the thermoalkaliphilic *Bacillus stearothermophilus* KGSHW-1 and showed the maximum lipase activity (15024.67 U/mL) in the culture medium supplemented with different nitrogen sources. A good yield of lipase could be obtained by supplementing the preferred nitrogen sources with ammonium (Rohit et al., 2001).

Gupta et al. (2004) and Christakopoulos (1992) also reported that ammonium chloride and ammonium sulphate are the best nitrogen sources for lipase production. In contrast, Pogaku et al. (2010) reported that potassium nitrate was found to be better than ammonium sulphate for maximum lipase production by *Staphylococcus* sp. LP12. Some other inorganic nitrogen sources such as sodium nitrate have also been reported to be effective in lipolytic activity by some microorganisms (Sharma et al., 2011). Some lipolytic fungi isolated from oil-spilled soil showed that among the various types of nitrogen sources, urea was the best nitrogen source to enhance the lipase production (2.35 U/mL), followed by ammonium chloride (2.05 U/mL) and ammonium sulphate (2.00 U/mL) (Christianah et al., 2012). Sharma et al. (2001) reported similar study for both bacterial and fungal isolates. Babu and

Rao (2007) reported maximum lipase production in the presence of urea in case of *Yarrowia lipolytica* NCIM 3589.

2.12.6 Effect of trace elements on lipase production

Tan et al. (2003) suggested that metal ions could influence the enzyme production as well as when the medium was supplemented with sodium (Na^{+}), magnesium (Mg^{+2}), and potassium (K^{+}). Lee et al. (1999) reported that metal ions influence the enzyme activity, synthesis and secretion mechanism in *Colletotrichum gloeosporioides* isolated from oil seed. Rooney and Weatherley, (2001) reported that addition of Mg^{2+}, Fe^{2+} and Ca^{2+} in culture medium could increase the lipase production by several fold in thermophilic *Bacillus* sp. It has been reported that *Bacillus* sp. A 30-1 (ATCC 1841) required a complex culture medium containing Ca^{+2}, Mg^{+2}, Na^{+}, Co^{+2}, Fe^{+2}, K^{+}, Mn^{+2}, Mo^{+2} and Zn^{+2}. Whereas, extracellular lipase produces by *Bacillus* sp. A 30-1 (ATCC 53841) showed its maximum activity in the presence of a complex medium that contained $Ca^{+2,}$ $Mg^{+2,}$ Na^{+}, Co^{+2}, Cu^{+2}, Fe^{+2}, K^{+}, $Mn^{+2,}$ $Mo^{+2,}$ and Zn^{+2} (Wang et al., 1995).

Lin et al. (1995) reported that lipase production by *Pseudomonas pseudoalcaligenes* F-111 was enhanced when the culture medium was additionally provided Mg^{+2} with phosphate. This alkaline lipase was observed to be most active and stable in the alkaline range pH 6.0-10.0 and its optimal activity was found at 40°C. Wang et al. (1995) isolated a lipase producing bacterium from mineral-rich hot spring (Yellowstone National Park), which showed its optimum activity at pH 9.0 and at 60°C.

Sharon et al. (1998) isolated *P. pseudoalcaligenes* KKA-5 exhibiting maximum lipase production in the presence of 0.8 M concentration of Mg^{+2}. It was also observed that lipase activity declined by 50% in the absence of the Mg^{+2} (Sharon et al., 1998), but under Ca^{+2} supplemented condition the lipase activity was not affected. However, the presence of Ca^{+2} enhanced the enzyme production in the thermophilic *Bacillus* sp. RS-12 (Sidhu et al.,

1998a, b). Janssen et al. (1994) isolated a thermophilic *Bacillus* sp. which showed increase in the lipolytic activity by several fold in the presence of Mg^{+2}, Fe^{+2} and Ca^{+2} ions into the culture medium. Similarly, the findings of Pokorny et al. (1994) in *A. niger*, showed that enzyme activity was stimulated by Mg^{+2}. The extracellular lipase activity of *Acinetobacter Calcoaceticus* BD 413 increased when the cultivation medium was supplemented with Mg^{+2}, Ca^{+2}, Cu^{+2} and Co^{+2} (Kok et al., 1995). The lipase dependent hydrolysis of p-nitrophenol (pNP) esters was better in between pH 7.8 to 8.8. The extracellular lipase from *Aspergillus calcoaceticus* showed result comparable with *Pseudomonas* lipases. The maximum enzyme production was detected when cultivation medium was supplemented with $MgSO_4$ followed by $FeSO_4$ (Bisht et al., 2012).

Kulkarni and Garde, (2002) isolated *P. aeruginosa* where in Ca^{+2} in addition to Mg^{+2} stimulated the enzyme production and activity. Gupta et al. (2004) reported that calcium and magnesium ions showed the best stimulatory effect on *Burkhloderia* sp. lipase production. Similarly, Gupta et al. (2004) also reported that Fe^{+2} plays a critical role in the production of extracellular lipase by *Pseudomonas* sp. Sangeetha et al. (2011) reported that divalent ions had a stimulatory effect on extracellular lipase production. McKellar et al. (1986) revealed that Fe^{+3} repressed the lipase production and activity, while growth was fastest at 2 μM in *P. fluorescens* B52. While using up to 400 μM concentration of iron, it showed a little effect on the lipase production. *P. fluorescens* B52 was also found to produce a siderophore, pyoverdine, which facilitates iron uptake in low-iron growth conditions. Hence, addition of siderophores to *P. fluorescens* B52 stimulated the production of lipase.

2.13 Purification of lipase enzyme

The purpose of purification is not only to isolate enzyme from their crude sources, but it helps to improve the stability, activity and shelf-life of enzyme. When the enzymes are

purified up to homogeneity level, it is easier to detect their structural and conformational function (Nadeem et al., 2009, 2015). The kinetic and thermodynamic characteristics of microbial lipases during substrate hydrolysis and transesterification, the structure-function relationship can be studied only in purified lipases. The purification steps of microbial lipase depend on their intracellular or extracellular nature. Moreover, purified proteins also require some modification and formulation to maximize their efficiency.

The main restraints in traditional purification strategies include low yields with long duration. Alternative new technologies are available for purification such as aqueous two-phase systems, membrane processes and immune purification. For large-scale production of enzymes, the purification strategies should be low cost, rapid and high yielding. There are numerous strategies for purification that have been used and highlighted for purification of enzyme from different microbes (Palekar et al., 2000; Saxena et al., 2003; Singh and Mukhopadhyay, 2011). Silva and Meirelles (2001) described an effective and economical method for the separation and purification of lipase in an aqueous two-phase system (ATPS). It can be formed combining either two incompatible polymers or a polymer and salt in water, above a certain critical concentration. The ATPS system is more affordable due to low cost, rapid process and easily available separators, which makes the protein separation easier and faster.

Enzyme purification depends on nonspecific techniques such as precipitation, dialysis, hydrophobic interaction chromatography, gel filtration, and ion exchange chromatography. In the microbial cell enzyme purification, first step is to remove microbial cell from culture broth after the fermentation process to get extracellular lipases. The cell culture or broth is passed in column chromatography through different stages depending on the nature of protein and required level of purification. The other techniques such as anion exchange, cation exchange and size exclusion chromatography are used for the purification of lipases.

After the microbial cell removal, enzymes are salted out by using ammonium sulphate by precipitation technique (Yang et al., 2016). Ammonium sulphate precipitation technique is mostly used at primary stages of purification, considered as a crude separation step and is followed by a combination of chromatographic steps (Saxena et al., 2003). In the next step, precipitated enzymes protein is subjected to dialysis and chromatography technique. The chromatography techniques are used according to the source and size of the microbial enzyme. Woolley and Peterson, (1994) reported that in some cases of affinity chromatography the number of purification steps decreases. Microbial lipase is hydrophobic in nature and possess large hydrophobic surface around active site of enzyme, thus the purification can be achieved by hydrophobic interaction chromatography. The precipitation method often gives about 87% average yield with limited purification which can be used in the formulation of detergents. However, high purification level is needed for synthetic reaction in pharmaceutical, leather and food industry. After the precipitation, alternative method such as ion exchange and gel filtration are usually preferred because of costly hydrophobic matrices (Shu et al., 2006). The crude extract after the precipitation are DEAE-sepharose and gel filtration chromatography (Unni et al., 2016; Zhou et al., 2012). There are many previous studied on lipase purification by ion exchange chromatography (Sharma et al., 2001). Zheng-Yu et al. (2007) purified lipase from *A. niger* F04 by ion-exchange chromatography and Sepharose G-74 followed by ammonium sulphate precipitation. The average yield of 33% was obtained, where the purification factor was 73. A highly alkaline lipase was purified from *Proteus vulgaris* by ion-exchange chromatography. The purified lipase showed the maximum lipolytic activity at alkaline pH 10.0 and its molecular weight was 31 kDa as recorded by SDS-PAGE (Kim et al., 1996). Extracellular lipase from *P. aeruginosa* possess an average molecular weight of 29 kDa (Unni et al., 2016). Microbial lipase also exists as a monomer with average weight of 46

kDa in yeast as reported by Kumar et al. (2014). Sugihara et al. (1995) reported that lipase from *Pichia burtonii* was purified by using many techniques including DEAE- Sephadex A-50 ion-exchange, Sephadex G-100 and isoelectric focusing. The purified lipase possesses monomeric nature and its molecular weight was estimated to be 51 kDa by SDS-PAGE.

Chartrain et al. (1993), purified a bacterial lipase from *P. aeruginosa* MB5001 by concentration it by ultrafiltration was followed by ion-exchange and gel filtration chromatography. The SDS-PAGE technique was used to determine the molecular mass that was found to be 29 kDa. However, Sharon et al. (1998) had also purified extracellular lipase from *P. aeruginosa* KKA-5 by ammonium sulphate precipitation and hydroxyapatite. The enzyme was found homogenous after a 518-fold of purification and molecular weight was estimated to be 30 kDa.

Lee et al. (1999) applied a three-step procedure including ammonium sulphate precipitation, DEAE-Sephacel and Sephacryl S-200 gel filtration chromatography for purification of lipase from a thermophilic *Bacillus thermoleovorans* ID-1. The protein was found homogenous and concentrated 223-fold and molecular mass 34 kDa estimated by SDS-PAGE method. Simons et al. (1998) reported a lipase from *S. epidermidis* RP 62A, which was purified to homogeneity by ammonium sulphate precipitation, affinity and gel filtration chromatography. The purified lipase showed its maximum catalytic activity at pH 6.0 in the presence of calcium as a cofactor. A bacterial lipase was purified from *Penicillium simplicissimum* after successfully following three-step procedure containing precipitation by ammonium sulphate, phenyl-SepharoseCL-4B, Ultrogel AcA-54 and chromatographic separation on hydroxyl appetite. The molecular weight was detected as 56,000 $gmol^{-1}$ with 20% recovery (Sztajer et al., 1992).

Ohnishi et al. (1994b) reported that *Aspergillus oryzae* could produce two kinds of extracellular lipase L1 and L2. Lipase L1 was purified to homogeneity by ammonium sulphate precipitation and acetone fraction. The purified enzyme was a monomeric protein of 24,000gmol^{-1} molecular weight which cleaved all the ester bond of triolein.

Shimada et al. (1993) isolated a lipase producing fungi *Fusarium heterosporum* and purified lipase with the recovery of 38% yield by SP-Sephadex chromatography, gel filtration, and isoelectric focusing. It was found that lipase has a monomeric protein and its molecular weight was estimated to be 31,000 gmol^{-1} by SDS-PAGE. Similarly, Yadav et al. (1998) had also purified lipase from *Aspergillus carneus* by two-step procedure involving precipitation process and hydrophobic interaction chromatography on octyl-Sepharose. The molecular mass was estimated by SDS-PAGE aas 27,000gmol^{-1}. Mayordoma et al. (2000) have also purified lipase from *Aspergillus nidulans* WG312 by phenyl-Sepharose chromatography and affinity binding on linolenic acid agarose with 29000 gmol^{-1} molecular weight.

Hiol et al. (2000) purified an extracellular lipase from *Rhizopus oryzae* by precipitation through ammonium sulphate, sulfopropyl sepharose chromatography, Sephadex G-75 gel filtration, and a second sulfopropyl sepharose chromatography. Lipase was purified 1200-fold with 32 kDa of molecular weight and isoelectric point at pH 7.6. Nawani and Kaur (2000) reported a thermostable lipase from *Bacillus* sp. which was purified to 175-fold by ammonium sulphate precipitation and phenyl Sepharose column chromatography. The recovery was found to be as 15.6 % and lipase showed a monomeric protein of 45 kDa molecular weight which hydrolysed all the ester bond of triolein.

2.14 Characterization of lipase

The physiochemical properties of recently studied lipase from bacterial source predicted

that all the bacterial lipases except that from *Halobacillus sp.* strain LY5 (Xin et al., 2012) and *Pseudomonas gessardii* (Ramani et al., 2010) lipase have 70 kDa molecular weight. The minimum molecular mass of lipase was observed as 19 and 19.2 kDa from *Bacillus stratosphericus* (Gricajeva et al., 2016) and *Enterococcus faecium* (Ramakrishnan et al., 2016), respectively.

The effect of pH on the lipase activity was studied over a wide pH range 3.0-12.0 in *P. aeruginosa* KM110. The results suggested that lipase was the most active at pH 6.0 and 9.0. The enzyme activity gradually increased from 7.0 -9.0 pH and it was characterized as an alkalophilic enzyme. The nature of the *P. aeruginosa* KM110 lipase proved it to be a potential alkaline lipase (Mobarak-Qamsari et al., 2011). Gupta et al. (2004) had reviewed the properties of bacterial lipase and it revealed that most of the bacterial lipase showed their maximum activity at a higher pH 7.0. Some bacterial lipase from *Pseudomonas gessardii, Spirulina platensis* and *Bacillus pumilus* RK31 showed maximum activity at acidic pH, while all other bacterial lipases worked at alkaline pH. Lipase from *P. fluorescens* SIK W1 also showed optimum activity at pH 4.8. Lee et al. (1999) studied the properties of extracellular lipase from *Bacillius thermoleovorans* ID-1 which showed its optimal activity at pH 7.5 and 70-75°C and retained its maximum activity at 60°C after 60

min of incubation. Golaki et al. (2015) studied the effect of temperature on *Cohnella* sp. A01 lipase which showed its maximum activity at 70°C, while all other bacterial lipase showed optimal activity below 70°C. Cold active lipase from *Acinetobacter* sp. XMZ-26 showed optimum activity at 15°C temperature (Zheng et al. 2011). Some other reports also revealed that lipase from *P. fluorescens* HU380 (Kojima and Shimizu, 2003), *Pseudomonas mendocina* PK-12CS (Jinwal et al., 2003) and *P. fluorescens* 2D (Makhzoum et al., 1996) have pH optima of 8.5, 8.0, 8.5 and 9.0, respectively. Lipases from *P. pseudomalei* 12 (Kanwar and Goswami, 2002) and *P. aeruginosa* YS-7 (Shabtai and Daya-Mishne, 1992)

growing in different water restricted environments are stable within the pH ranges of 7-10.5 and 6.5-7.5, respectively.

Sztajer et al. (1992) showed that *Pseudomans simplicissimum* lipase has low stability in water-miscible organic solvents with good stability in water immiscible solvents such as hexane, heptane, benzene and isooctane. Bussamara et al. (2010) reported that the lipase activity in yeast *Pseudomonas hubeiensis* was enhanced up to 123.4% by EDTA, while Ca^{2+} showed its inhibitory effects on the enzyme activity. In case of *C. verticillata* lipase, the enzyme activity was strongly inhibited by $AgNO_3$, $NiCl_2$, $HgCl_2$, $CdCl_2$ and EDTA; while the presence of $Ca^{2+,}$ Mn^{2+} and Ba^{2+} ions enhanced the activity of the enzyme (Gopinath et al., 2002).

Lipases are able to work in both organic solvents and in aqueous solutions. Their stability in organic solvents is desirable especially in reactions involving synthesis. Various water-miscible and water-immiscible solvents were tested for their effects on lipase activity and stability. A marked stimulation in the enzyme activity was observed upon addition of hexane up to 80% to the assay mixture. In contrast, ethanol, methanol and isopropanol inhibited the enzyme at concentrations of 50% and above, but no effects were observed at 20 % in all the solvents (Shailu et al., 2012). Thus, water miscible solvents (ethanol, acetone and isopropanol) appeared to be concentration dependent, where low concentration of these solvents showed some stimulatory effects on the lipase activity. Similar trend in stability was observed in *P. chrysogenum* 9' lipase, where the enzyme was more than 90% stable in the presence of hexane, 1,4 dioxane and almost 70 % in the presence of cyclohexane, butanol, pentanol and xylene inhibited the enzyme activity completely at higher concentrations (Bancerz et al., 2005).

CHAPTER 3

CHAPTER 3

ISOLATION, SCREENING, BIOCHEMICAL CHARACTERIZATION OF EXTRACELLULAR LIPASE PRODUCING EXTREMOPHILIC BACTERIAL STRAINS

3.1 Introduction

Lipases are produced by numerous microorganisms as well as by various eukaryotes, which catalyze the hydrolysis and synthesis of long-chain acylglycerols (Ferrato et al., 1997; Sharma et al., 2001; Sachan and Singh 2015). Lipases constitute the most important group of biocatalysts for biotechnological applications. They have conserved 3D structures but lack a high-sequence homology and display a wide diversity in properties. Due to their multiple commercial applications, there is need to search for novel lipases producing microorganisms. The special stability and activity ranges of lipases from extremophiles, is different from most of the mesophiles. Lipases from extremophiles offer easy and simple purification steps by employing the extreme conditions (Salameh et al., 2007).

Extremophiles are organisms, which are able to thrive at extremes of temperature, pressure, low water activity, salinity, acidity, or alkalinity and are also resistant to exposure of high radiation, pressure, metal concentrations or organic solvents. Extremophiles are not only found in niches exhibiting these conditions, but several strains have been isolated from non-extremophilic environment (Scandurra et al., 1998; Wiegel, 1998, Satyanarayana et al., 2005). Extremophiles have the potential to produce valuable biocatalysts that function under the unique conditions in which usually the enzymes of their non-extremophilic counterparts do not function. However, limited number of lipases that function at extreme conditions have been isolated from mesophilic origin, for example, thermophilic lipase from *Burkholderia cepacia* (Rathi et al., 2002), acidophilic and thermophilic lipase from

Kurtzmonomyces sp. I-11 (Kakugawa et al., 2002), and alkaliphilic lipase frorn *B. subtilis* (Lesuisse et al., 1993, Emtenani et al., 2013).

Lipases from halophiles including from the novel group of haloalkalithermophiles could be of great interest. The alkaliphilic lipases have been reviewed in the past (Horikoshi 1999., Jaeger and Eggert, 2002).

3.2 Materials and methods

3.2.1. Collection of samples

The soil and water samples were collected from agricultural soil of Leh, Laddakh (34.1526° N, 77.5771° E) and coastal Dwaraka (28.5823° N, 77.0500° E) Gujrat, (India) respectively. Soil samples were collected from a depth of 5 cm and placed in a sterile autoclaved bag. The soil sample dried at room temperature and grounded with a pestle-mortar and then sieved through a 2 mm sieve. The sieved sample was stored in refrigerator for further analysis. The water sample was preserved at 4°C for isolation and screening of bacterial isolates.

3.2.2. Isolation of bacterial strain

The extremophilic bacteria were isolated from soil and water sample collected from agricultural soil of Leh, Laddakh and water sample from Dwaraka Gujrat, (India) respectively. For the isolation of psychrotolerant bacteria serial dilution method was performed. A stock solution was prepared by mixing 10 g of the soil samples in 100 ml distilled water containing 1% NaCl solution. After preparing the stock, the dilution from 10^{-1} to 10^{-5} was prepared. The bacteria were isolated using nutrient agar (NA). The NA medium contained: g/L: Beef extract 1.5, Yeast extract 1.5, Peptone 5.0, NaCl 5.0, Agar 20.0, pH 7.2 ± 0.2.

For the isolation of halotolerant bacteria, 1 mL of saline water was inoculated in 50 mL of

nutrient broth in 250 mL Erlenmeyer flasks. The inoculated culture medium was kept on orbital shaker at 30°C for 48-72 hr at 150 rpm.

3.2.3. Molecular identification of isolated lipolytic microorganisms

The pure monoclonal colonies of the isolated extremophilic bacterial strain producing extracellular lipase were selected for identification. The culture of bacteria was sent to CSIR-NCL (National Chemical Laboratories), Pune, India for molecular identification. Molecular identification has been carried out by using 16S rRNA gene sequencing (Clarridge, 2004). The chromosomal nucleic acid extraction protocol was performed as described by Wilson et al. (2001). The DNA was extracted by using column kit (Hi Media, India) and 16S rRNA gene sequencing by Sanger's method, i.e. ABI 3500xL genetic analyzer. The 16S rRNA sequence of the bacteria was amplified by using bacteria specific universal primer 27F (5′- CCAGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′- CGGTTACCTTGTTACGACTT- 3′). The sequence was compared with 16S rRNA gene database using BLAST. The sequence data was submitted to NCBI. The phylogenetic tree of the isolated strain was constructed by using MEGA- X with the neighbour joining method. The isolated psychrotolerant strain was identified as *Pseudomonas punonensis* with accession number MH393314. The isolated halotolerant strain was identified as *Bacillus gibsonii*. It was given an accession number MK835660 and both the gene sequence have been submitted to the Genbank Data Library.

3.2.4. Estimation of salt concentration for optimum growth of halotolerant bacterial strain

The isolated halotolerant bacterial strain obtained from the costal saline water was optimized under different growth conditions. The optimum salt concentration for growth of isolated bacterium was found to be 600 mM, which was determined by inoculating the bacterial culture (1% inoculum) in the culture medium containing additional NaCl

concentration range from 50 to 1200 mM. The culture flasks were incubated at 30°C at 150 rpm for 120 hr. The sample was collected every 8 hr up to 120 hr for estimating the optical density of the culture at 600 nm using double beam (UV-Vis spectrophotometer UV-1601, Shimadzu, Japan). The temperature tolerant profile of psychrophilic bacterium was determined by incubating the bacterial culture under different temperature conditions (4-35°C) under shaking condition. The bacterial growth was formed to be maximum at 16°C.

3.2.5. Morphological characterization of isolated bacterial strains

After purification of bacteria on NA plates, the morphological study was carried out by using 48 hr old culture. Morphological feature of colony was recorded under bright microscope.

Gram Staining is a common technique used for identification of bacterial cells. Danish Bacteriologist Hans Christian Gram introduced this technique in 1884. This technique is useful in the differentiation of the bacteria into Gram-Positive and Gram-Negative bacteria, which helps in the classification and differentiations of bacteria.

A) Gram Staining

The cell walls of gram-positive bacteria have a thick layer of protein-sugar complexes called peptidoglycan and lipid content is low. Decolorizing the cells causes this thick cell wall to dehydrate and shrink, which closes the pores in the cell wall and prevents the stain from exiting the cell. So the use of ethanol to wash the cells cannot remove the Crystal Violet-Iodine complex that is bound to the thick layer of peptidoglycan of Gram positive bacteria and appears in blue or purple colour. In case of Gram negative bacteria, cell wall also takes up the Crystal Violet-Iodine complex but due to the thin layer of peptidoglycan and thick outer layer which is formed of lipids, Crystal Violet-Iodine complex gets washed off with ethanol as it dissolves the lipids in the cell walls, which allows the crystal violet-

iodine complex to leach out of the cells. Then when again stained with safranin, they take the stain and appears reddish or pinkish in color.

Materials required:

- Glass slide
- Inoculating loop
- Immersion oil
- Distilled water
- Microscope
- Bunsen burner

Chemical required:

- Crystal violet
- Iodine
- A decolorizer made of acetone and alcohol (95%)
- Safranin, the counterstain

Procedure of Gram Staining

1. A clean glass slide was taken and smear of isolated bacterial suspension were put on the slide with the help of inoculation loop.
2. We kept it at room temperature for allow it to dry and fix it with flame.
3. After that, Crystal Violet was poured and kept for about 30 to 60 seconds and smear was rinsed with distilled water gently.
4. Next, Gram's iodine was poured for 60 seconds and wash with distilled water.
5. Again, the smear was washed with 95% (v/v) alcohol or acetone for about 10-20 seconds and again rinsed with water.
6. Safranin was poured for 60 seconds and again wash with distilled water.

7. We observe the dried slide under Microscope.

Interpretation

Gram negative bacteria show pink colour stain while Gram positive show purple stain.

3.2.6 Screening by biochemical tests

A) Casein hydrolysis for protease enzyme (Larsen et al., 1998)

Some microorganisms have the ability to degrade the casein protein by producing proteolytic exoenzyme, called proteinase (caseinase). For the screening of such an activity skimmed milk agar is used. The medium is composed of nutrient agar supplemented with skimmed milk that contains the proteinase substrate casein.

Chemicals:

Skimmed milk agar powder 28.0 g/L,

Tryptone 5.0 g/L,

Yeast extract 2.50 g/L,

Dextrose (Glucose) 1.0 g/L,

Agar 15.0 g/L,

Final pH 7.4±0.2 (at 25°C)

Method:

- After preparing Skimmed milk agar media, the isolated bacteria were inoculated on skimmed milk agar plate by streaking.
- The plates were incubated at 25°C for 48 hr to examine the hydrolysis.
- After incubation, the positive result indicates the clear zone around bacteria.

B) Starch hydrolysis (Iverson & Millis 1974)

Starch is a complex polysaccharide found in plants is usually deposited in the form of large

granules in cytoplasm of the cell. Starch consists of 2 components—amylose and amylopectin, which are present in various amounts. The amylose consists of D-glucose units linked in a linear fashion by α-1,4 linkages. It has 2 non-reducing ends and a reducing end.

Amylopectin is a branched polysaccharide. In these molecules, shorter chains of glucose units linked by α-1,4 glycosidic bond with other residue joined to each other by α-1,6 linkages. In the starch hydrolysis test, the test bacteria are grown on agar plates containing starch. If the bacteria have the ability to hydrolyze starch, particularly in the areas surrounding their growth, whereas rest of the area on the plate still contain non-hydrolysed starch.

Since no color change occurs on plate where microorganism has hydrolyzed the starch, iodine solution is added to the plate as an indicator of starch hydrolysis after incubation whereas, the non-hydrolysed starch forms dark blue color with iodine, its hydrolyzed end products do not acquire such dark blue color with iodine.

Chemicals

Starch agar

Peptic digest of animal tissue 5.0 g/L,

Sodium chloride 5.0 g/L,

Yeast extract 1.5 g/L,

Beef extract 1.5 g/L

Starch soluble 2.0 g/L

Agar 15.0 g/L

Final pH (at 25°C) 7.4±0.2

Method

- The isolated bacteria were inoculated with the help of inoculation loop on Starch agar plate.
- After inoculation, the plates were incubated for 48 hr at 37°C to examine the starch hydrolysis.
- After incubation, the plates surface was flooded by iodine solution with a dropper for 30 seconds.
- We pour off the excess iodine and observe the hydrolysis. Starch hydrolysis was detected by clear zone around the bacterial growth.

C) Citrate utilization test (Ishiguro & Sato 1979)

Citrate agar is used to test an organism's ability to utilize citrate as a source of carbon and energy. The NA medium contains citrate as the sole carbon source and inorganic ammonium salts ($NH_4H_2PO_4$) as the sole source of nitrogen.

Bacteria grown on this medium produce an enzyme, citrate-permease, capable of converting citrate to pyruvate. Pyruvate can then enter the organism's metabolic cycle for the production of energy. Growth is indicative of utilization of citrate as an intermediate metabolite in the Kreb's cycle. When the bacteria metabolize the citrate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH (above 7.6) turns the bromthymol blue indicator in the medium from green to blue.

Chemicals:

Sodium Chloride 5.0 g/L

Sodium Citrate (dehydrate) 2.0 g/L

Ammonium Dihydrogen Phosphate 1.0 g/L

Dipotassium Phosphate 1.0 g/L

Magnesium Sulphate (heptahydrate) 0.2g/L

Bromothymol Blue 0.08 g/L

Agar 15.0 g/L

Final pH 6.9 ±0.2 (25°C)

Media preparation

- The media was prepared by dissolving above chemicals and salts in distilled water. The pH was adjusted to 6.9 by 0.1 N NaOH.
- After adjusting pH, Bromothymol blue and agar was added.
- The media was heated to boiled until agar was dissolved.
- The slant was prepared with 4.0 to 5.0 ml of media into 16-mm test tubes.
- The slant tubes were autoclaved at 121°C under 15 psi pressure for 15 minutes and allowed to cool in slanting position.
- A loopful bacterial culture was inoculated in slant tubes.
- The uninoculated medium shows a deep forest green due to the pH of the sample and the bromothymol blue.
- The slants were incubated at 25°C for up to 48 hr to observe citrate utilization.
- A colour change from green to blue was considered a positive test for starch hydrolysis.

D) Triple sugar iron test (TSI) (Kolmos & Schmidt 1987)

The triple sugar- iron (TSI) agar test is designed to differentiate among organisms based on the differences in carbohydrate fermentation patterns and hydrogen sulfide production. Carbohydrate fermentation is indicated by the production of gas and a change in the colour of the pH indicator from red to yellow. TSI Agar contains three fermentative sugars, lactose and sucrose in (1% each) concentrations and glucose (0.1% w/v). The acid base indicator Phenol red is added for detecting carbohydrate fermentation due to change in color of the carbohydrate medium from orange red to yellow in the presence of acids. In case of oxidative decarboxylation of peptone, the resulting alkaline products raise the pH which

changes the colour of the medium from orange red to deep red. Sodium thiosulfate and ferrous ammonium sulfate present in the medium detect the production of hydrogen sulfide which is indicated by the black color in the butt of the tube. To facilitate the detection of organisms that only ferment glucose, the glucose concentration is kept at one-tenth of the concentration of lactose or sucrose. The meagre amount of acid production in the slant of the tube during glucose fermentation oxidizes causes the medium to remain orange red or revert to an alkaline pH. In contrast, the acid reaction (yellow) is maintained in the butt of the tube since it is under lower oxygen tension. After depletion of the limited glucose, organisms begin to utilize the lactose or sucrose. To enhance the alkaline condition of the slant, free exchange of air must be permitted by closing the tube cap loosely.

Media:

TSI Agar

Enzymatic digest of casein 5 g/L
Enzymatic digest of animal tissue 5 g/L
Yeast enriched peptone 10 g/L
Dextrose 1 g/L
Lactose 10 g/L
Sucrose 10 g/L
Ferric ammonium citrate 0.2 g/L
NaCl 5 g/L
Sodium thiosulfate 0.3 g/L
Phenol red 0.025 g/L
Agar 13.5 g/L
pH 7.3 ±0.2 (25°C)

Method

- TSI media was prepared by above chemicals and salts.

- The bacteria were inoculated into the TSI by streaking the surface of the agar slant.
- The slant cap was leave slightly loose and incubate the tube at 25°C for 24 hr.
- After incubation period, the result was observed by change in colour of the media.

Result

- An alkaline/acid (red slant/yellow butt) reaction indicates dextrose fermentation only.
- An acid/acid (yellow slant/yellow butt) reaction indicates the fermentation of dextrose, lactose and/or sucrose.
- An alkaline/alkaline (red slant, red butt) reaction shows the absence of carbohydrate fermentation results.

E) Urease test (Seeliger 1956)

Urea Agar test was developed by Christensen in 1946 for the differentiation of Enteric bacilli. The urease test is used to determine the ability of an organism to split urea, through the production of the enzyme urease. Urea is the product of decarboxylation of amino acids. Hydrolysis of urea produces ammonia and CO_2. The formation of ammonia induce alkalinity in the medium, and the pH increase is detected by the colour change of phenol red from light orange (pH 6.8) to magenta pink (pH 8.1). Rapid urease-positive organisms turn the entire medium pink within 24 hr.

Chemicals:

Urea 20.0 g/L

Sodium chloride 50.0 g/L

Monopotassium Phosphate 2.0 g/L

Peptone 1.0 g/L

Dextrose 1.0 g/L

Phenol Red 0.012 g/L

Agar 15.0 g/L

pH 6.7 ±0.2 (25°C)

Preparation

- Urease agar media was prepared by dissolved all the above chemicals except agar in 100 ml of distilled water and filter it with 0.45-mm pore size.
- The agar was added in 900 ml of distilled water separately and boiled to dissolve completely.
- The prepared medium was autoclaved at 121 °C and 15 psi for 15 minutes and allowed it to cool up to 50 to 55°C.
- After that, 100 ml of filter-sterilized urea was added aseptically to the cooled agar solution and mix thoroughly.
- The slants were prepared by 4 to 5 ml medium added to sterile test tubes which was placed in slanting position until medium was solidified.

Procedure of Urease Test

- The isolated bacteria were streaked on the surface of urea agar slant with inoculation loop.
- The loosely capped slants tubes were incubated at 35-37°C in ambient air for 48 hr to 72 hr.
- The result was examined by change in slant colour which development a pink colour for as long as 72 hr.

F) Lipase production (Kumar et al., 2012)

Screening of Lipase production by isolated bacteria was carried out using nutrient agar media with 1% tributyrin as carbon source for the production of extracellular lipase by

bacteria which hydrolyze the tributyrin and produces a clear zone. The bacterial colonies showing a halo zone on this medium were considered lipase positive.

Chemicals:

Peptone 5.0 g/L

Yeast extract 1.5 g/L

Beef extract 1.5 g/L

Sodium Chloride 5.0 g/L

Agar 15.0 g/L

Tributyrin 1%

Final pH 7.2 ±0.2 (25°C)

Procedure:

- Tributyrin media was prepared by adding it in nutrient agar media.
- The isolated bacteria were streaked on the surface of agar media by inoculation loop.
- The streaked plates were incubated at 25°C for 48 hr to examine the hydrolysis of tributyrin.
- The formation of halo zone was considered as lipase positive activity.

G) Lignin peroxidase activity

Azure B agar plate assay (Archibald 1992)

Several microorganisms, which secrete lignin peroxidase and manganese peroxidase, show the ability to decolorize or degrade Azure B dye. However, laccase enzyme cannot decolorize Azure B and therefore, thus, it can be used as a qualitative test for production of lignolytic peroxidase production enzyme.

Chemicals:

Minimal salt medium

Azure B dye

Glucose solution 20% w/v

Agar 15.0 gm/L

Procedure:

- 100 mL the minimal salt medium was supplemented with 0.01% Azure B dye.
- 1 ml of 20% (w/v) of aqueous glucose solution was added to 100 mL of medium.
- The medium was autoclaved at 121°C for 15 min and, then it was transferred to the plates.
- The plates were inoculated by isolated bacteria and incubated at 27°C under dark conditions.
- The plates were examined after 24 hr to examine the activity. The appearance of clear halo zones around bacterial colonies confirmed the lignolytic enzyme production.

3.2.7 Molecular characterization of bacterial isolates

The pure culture of the isolated strains producing extracellular lipase were sent to CSIR-NCL (National Chemical Laboratories), Pune, India for identification of bacterial strains. The 16S rRNA sequences of the bacteria were amplified by using bacteria specific universal primer 27F(5′-CCAGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT- 3′). Sequence were compared with 16S rRNA gene database using BLAST. The sequence data was submitted to NCBI. The phylogenetic tree of the isolated strain was constructed by using MEGA- X with the neighbour joining method.

3.3. Results and discussion

3.3.1 Isolation of extremophilic bacteria

Two extremophilic bacteria were isolated from soil and water samples. The psychrotolerant bacterium was isolated from soil of dry desert region of Leh, Laddakh (India) and it was identified as *Pseudomonas punonensis*. The halotolerant isolate from coastal saline water of Dwaraka, Gujrat, (India) was identified as *Bacillus gibsonii*. Both the strains were isolated on NA plates. Abdulkadir (2012) isolated bacterial strains on NA plates to confirm the antibiotics production. Keeping in view habitat specific requirement of bacterial isolates, the psychrotolerant *P. punonensis* plates were maintained at low temperature and *B. gibsonii* plates were supplemented with 4% NaCl at room temperature.

3.3.2 Morphological characterization of bacterial isolates

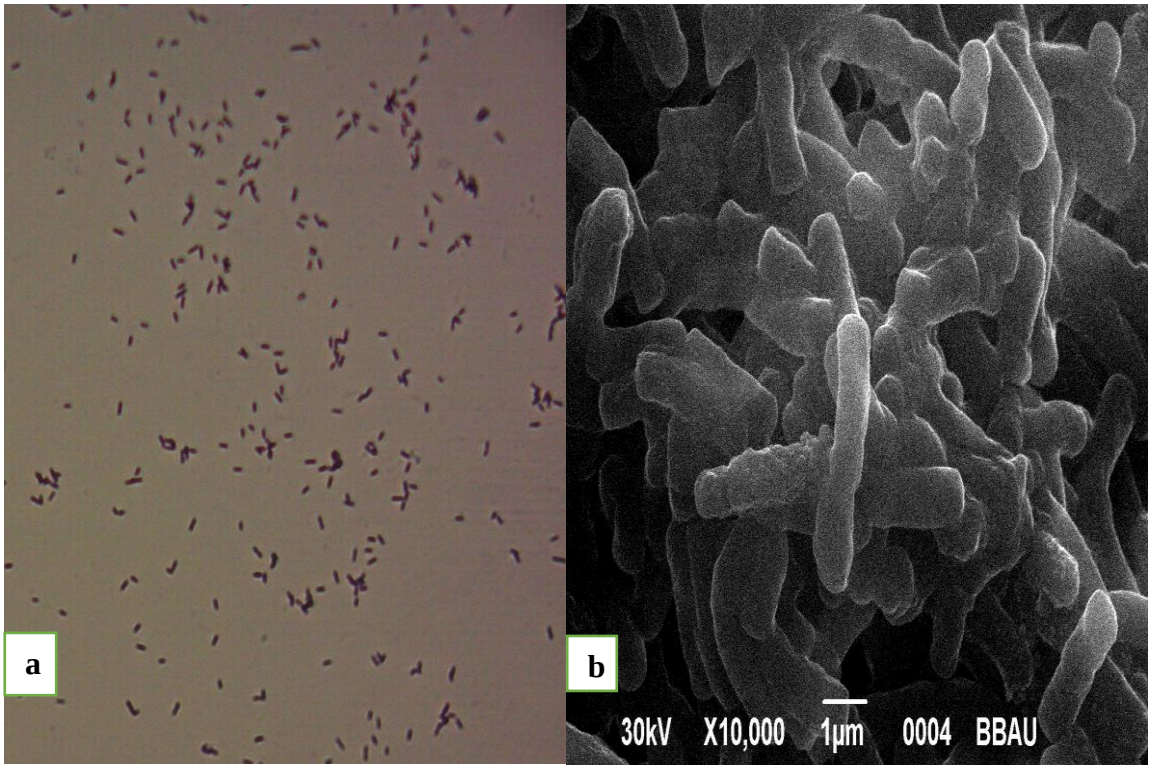
In the present study, examination of macroscopic features of psychrotolerant bacterial strains on NA plates showed moderate growth, yellow colored colony with velvet appearance. Whereas, on Kings B agar, the psychrotolerant strain exhibited faster growth, yellow shine like appearance. The pigment produced by the psychrophilic bacteria on Kings B media was yellow in colour. The colony of mesophilic halotolerant bacterial strain exhibited a light-yellow coloured colony on NA plates.

Colony morphology refers to visual characteristics of a bacterial colony. Bacterial colonies that differ in appearance are typically different bacterial strains, species, or genera. However, colony morphology is not the sole criteria for identification of bacteria, as many different types of bacterial strains have similar colony morphology. Gram staining of isolates revealed that extremophilic bacterial strains were Gram negative, rod shaped cells. Microscopic study using light microscope and Scanning Electron Microscope (Fig 3.1) revealed that average length of *P. punonensis* cell was 3.6 μm and width was 0.7 μm. However, the average length and width of *B. gibsonii* were 4.64 μm and 0.80 μm

respectively. Morphological observations revealed that both the strains have character such as rod shape and rough edge.

Table 3.1 Morphological characteristics of isolated bacterial strains

S.No.	Characteristics Morphology	*Pseudomonas punonensis*	*Bacillus gibsonii*
1.	Color	Dark yellow	Light yellow
2.	Gram stain	-ve	-ve
3.	Cell shape	Rod	Rod
4.	Surface texture	velvet	Rough
5.	Margin	Entire	Entire
6.	Elevation	Flat	Flat



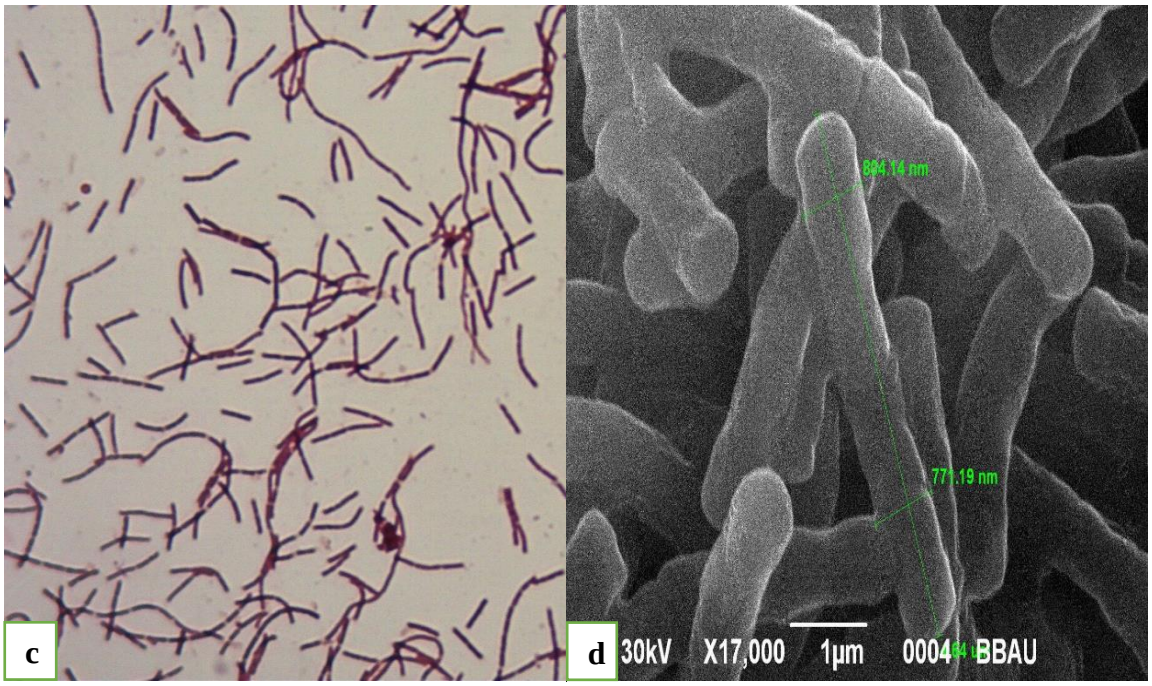


Fig 3.1 Morphological/microscopic identification of isolated psychrotolerant *Pseudomonas punonensis* (a,b) and halotolerant *Bacillus gibsonii* (c,d) by light microscope (Fig 3.1 a,c) and scanning electron microscope (Fig 3.1 b,d)

3.3.3 Molecular identification of isolated bacterial strain

The molecular identification of bacterial isolates was done by 16 S rRNA gene sequencing. The 16S rRNA sequences of the organism was amplified by using bacteria specific universal primer 27F (5´- CCAGAGTTTGATCCTGGCTCAG-3´) and 1492R (5´- CGGTTACCTTGTTACGACTT- 3´). Sequence were compared with 16S rRNA gene database using BLAST. The 1343 bp long rRNA segment for psychrotolerant and 1335 bp segment for halotolerant strains were purified and amplified by using Sanger method (Woese et al., 1976). Based on sequencing of 16S r RNA and BLAST analysis, the psychrotolerant *Pseudomonas* (MH393314) showed 99% similarity to *Pseudomonas punonensis* and the nearest strain was *Pseudomonas punonensis* strain LMT03. Halotolerant *Bacillus* (MK835660) was identified as *Bacillus* sp. with 98.48% homology. Based on the NCBI database, the form of phylogenetic tree as shown in fig 3.2.

A) Gene sequence of *Pseudomonas punonensis*

>F27_Seq53-*Pseudomonas punonensis*

CAGGGCGGCAGTCTACACATGCAGTCGAGCGGTTGACGGGAGCTTGCTCCCT
GATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTATTAGTGGGG
GACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGC
AGGGGACCTTCGGGCCTTGCGCTAATAGATGAGCCTAGGTCGGATTAGCTAG
TTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGA
TGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGC
AGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGT
GTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGT
AAATTAATACTTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACT
CTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTAC
TGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCCGG
GCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTACGGTAGAGGG
TGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACC
AGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCA
ACTAGCCGTTGGGTTCCTTGAGAACTTAGTGGCGCAGCTAACGCATTAAGTTG
ACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAGAAAATTTTGGAAC
GGGA

B) Gene sequence of *Bacillus gibsonii*

>F27_SEQ116_*Bacillus gibsonii*

CACAGCTACTCTATGATGCAGTCGAGCGGCACGTTTTTGAAGCTTGCTTCAA
AAACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTACCTTATCGA
CTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATCTAGCACCT
CCTGGTGCCGGATTAAAAGAGGGCTTCTTGCTCTCACGATGAGATGGGCCC
GCGGCGCATTAGCTAGTTGGAGAGGTAACGGCTCCCCAAGGCGACGATGC
GTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGT
CTGACGGAGCAACGCCGCGTGAGTGATGAAGGGTTTCGGCTCGTAAAGCTC
TGTTATGAGGGAAGAACACGTACCGTTCGAATAGGGCGGTACCTTGACGGT
ACCTCATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
TAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGG
CCTTTTAAGTCTGATGTGAAATCTTGCGGCTCAACCGCAAGCGGCCATTGG
AAACTGGGAGGCTTGAGTACAGAAGAGGAGAGTGGAATTCCACGTGTAGC
GGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTC
TGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGGT
TTCGATGCCCGTAGTGCCGAAGTTAACACATTAAGCACTCCGCCTGGGGAG
TACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
AGTGGAGCATGTGGGTTTAATTTCGAAGCAACGCGAAGAACCCTTTACCAG
GTCTTTGACATCCTTTGACCACTCTGGAGACAGAGCTTCCCCTTCGGGGGGG

CAAAGTGACAGGTGGTGCCAATGGTGTTCGTCAGCATCGATGTCGTGAGCA
TGCATGGGATTAGTTCCCGCAACAGAAGCCGCA

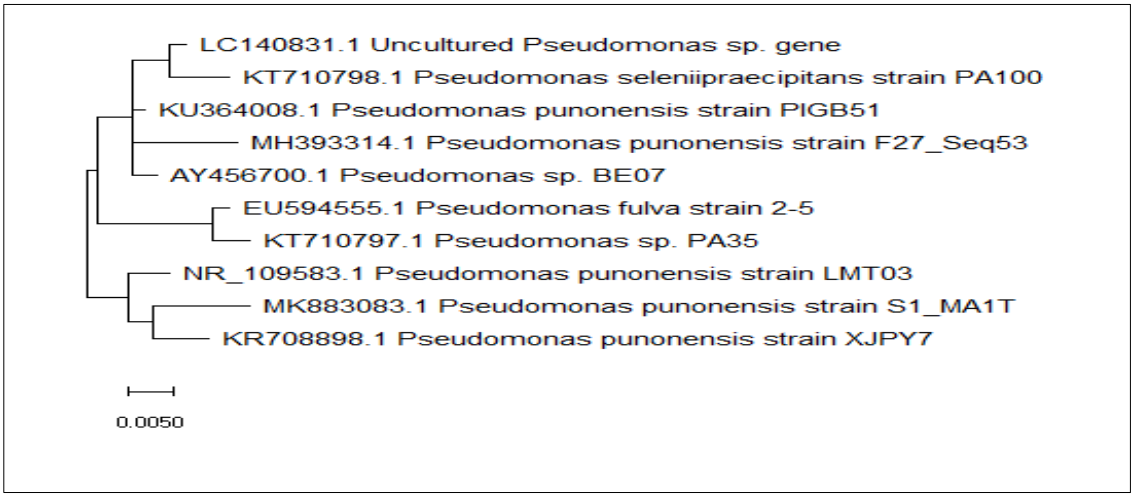


Fig. 3.2. (a) Phylogenetic tree based on 16S rRNA sequence of bacterial strain and representative species of the genus *Pseudomonas punonensis*

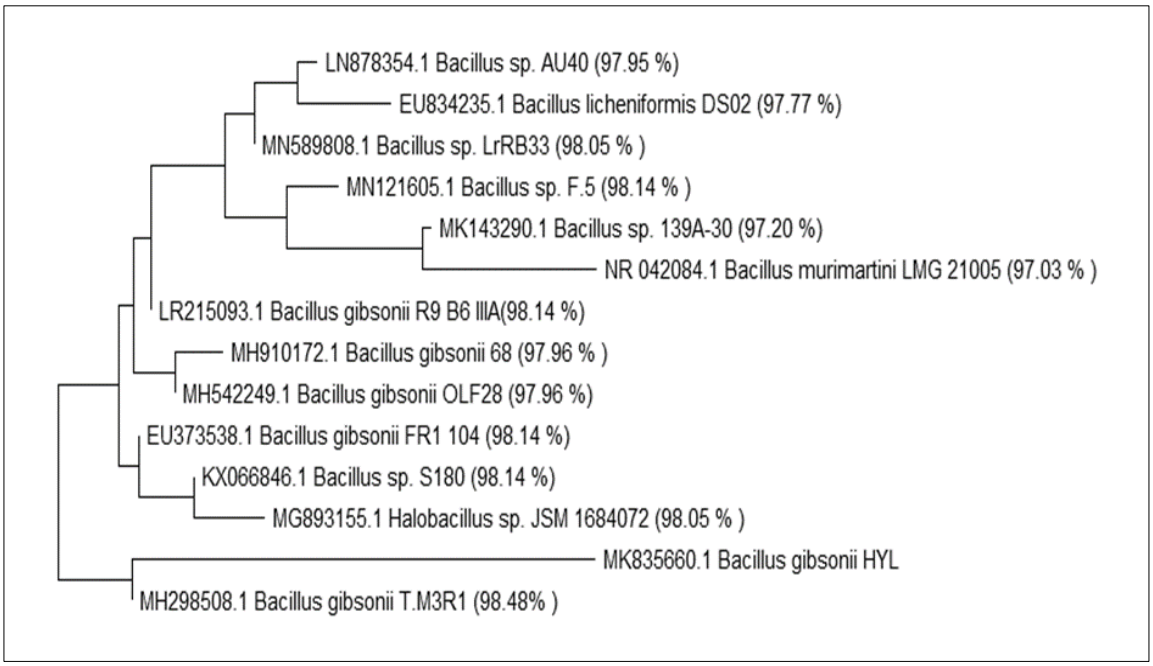


Fig.3.2. (b) Phylogenetic tree based on 16S rRNA sequence of bacterial strain and representative Species of the genus Bacillus *sp.*

3.3.4 Biochemical characterization of isolated bacteria

A) Starch hydrolysis

The amylases enzyme hydrolysed the glycosidic bonds at α-1, 4 and α-1,6 linkage of starch. The amylase is divided into α-amylases; the one which disrupts the inner bonds of the substrate (endoamylases) and β- amylases which breaks the non-reducing ends of the substrate (exoamylases) and the glucoamylases (amyloglucosidases), which releases glucose units from the non-reducing end of starch molecules (Gupta, et al., 2003; Norouzian, et al., 2006). In the starch hydrolysis test, both the exremophilic strains did not grow on starch as carbon source, which confirmed that these strains did not produce amylase enzyme.

B) Cellulase production

Teather and Wood (1982), showed strong interaction of congo-red with polysaccahrides with connecting P-(1-4)-bound-D-glucopyranosyl units which provided the basis for a sensitive assay for detecting colonies of cellulase producing bacteria. Later Sazci et al. (1986) used the Congo red to detect cellulase- producing fungi. In the present study, only halotolerant *B. gibsonii* showed a 0.5 cm clear zone around the bacterial colony grown on 1 % carboxy methyl cellulose (CMC) containing petriplates. However, psychrotolerant *P. punonensis* did not show the cellulase enzyme activity (Fig 3.3 a). Shankar & Isaiarasu (2011) studied the cellulase production by *B. pumilus* EWBCM1 isolated from earthworm gut. The maximum cellulase activity was observed at 37°C at pH 6.0. Ray et al. (2007) studied on different factors which affect the cellulase production in *B. subtilis* CY5 and *B. circulans* TP3 isolated from fish gut. Acharya and Chaudhary (2012) studied the cellualse

production from thermotolerant *B. licheniformis* MVS1 and *Bacillus* sp. MVS3 isolated from Indian hot spring.

C) Triple Sugar iron test

The triple sugar- iron agar test employing triple sugar iron agar is designed to differentiate among organisms based on the differences in carbohydrate fermentation patterns and hydrogen sulfide production. Carbohydrate fermentation is indicated by the production of gas and a change in the colour of the pH indicator from red to yellow.

Interpretation

- An alkaline/acid (red slant/yellow butt) reaction: It is indicative of dextrose fermentation only.
- An acid/acid (yellow slant/yellow butt) reaction: It indicated the fermentation of dextrose, lactose and/or sucrose.
- An alkaline/alkaline (red slant, red butt) reaction: Absence of carbohydrate fermentation results.

Based on the result as shown in fig 3.4(a) both the extremophilic strains showsed red slant and red but which confirmed the absence of carbohydrate fermentation.

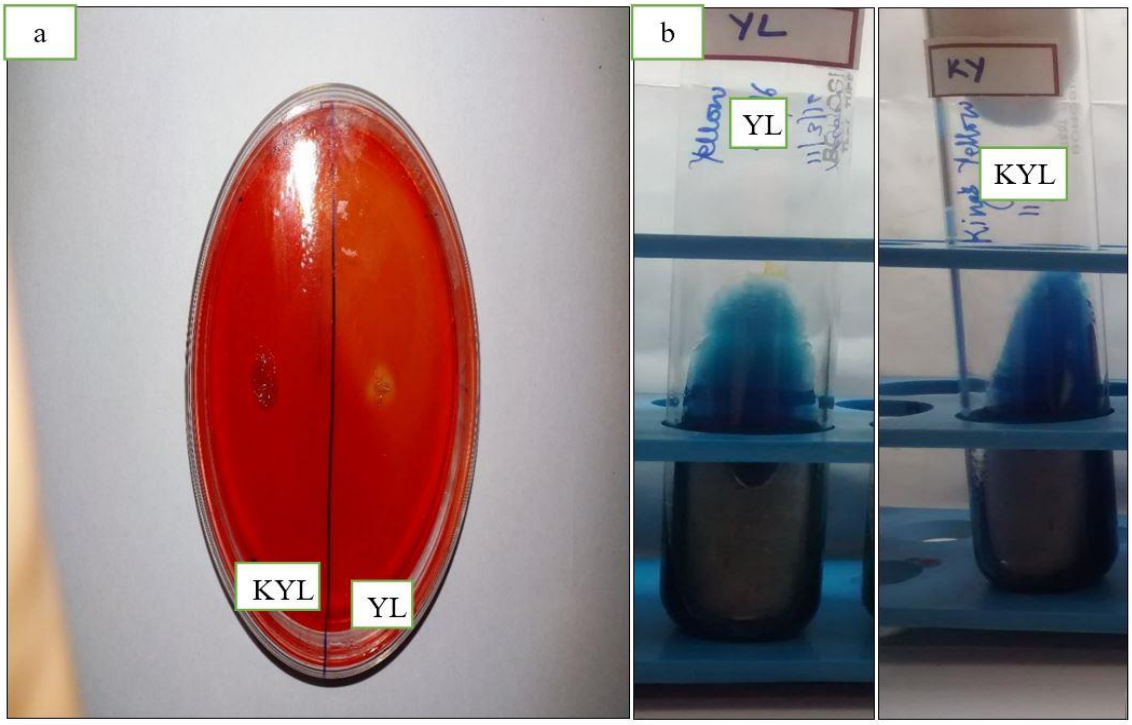


Fig.3.3. Cellulase (a) and Citrate utilization (b) production by *Pseudomonas punonnensis* (KYL) and *Bacillus gibsonii* (YL)

D) Urease test

Urease test is a biochemical test that detects the alkaline fermentation of urea with the resultant production of ammonia by microorganisms. The positive result is predicted by change in the media colour from yellow to pink. Those microorganisms which produce ureases, utilize urea as a sole nitrogen source and they may produce a sufficient amount of ammonia to overcome the buffering capacity of the medium. The colour of media change as a result of change in pH. The result shown in fig. 3.4(b) revealed that both the extremophilic strains produce urease enzyme.

Goswami et al., (2015) studied on plant growth promoting rhizobacterium (PGPR) strain of *P. aeruginosa* for production of urease. Patel et al. (2013) studied on characterization of urease producing *P. fluorescens*. Two urease producing *Bacillus* sp. Were isolated from highly alkaline cement samples (Achal et al., 2010). Sarda et al. (2009) reported that

induced biocalcification or calcite preparation involve the activity of urease enzyme in *Bacillus pasteurii* NCIM 2477.

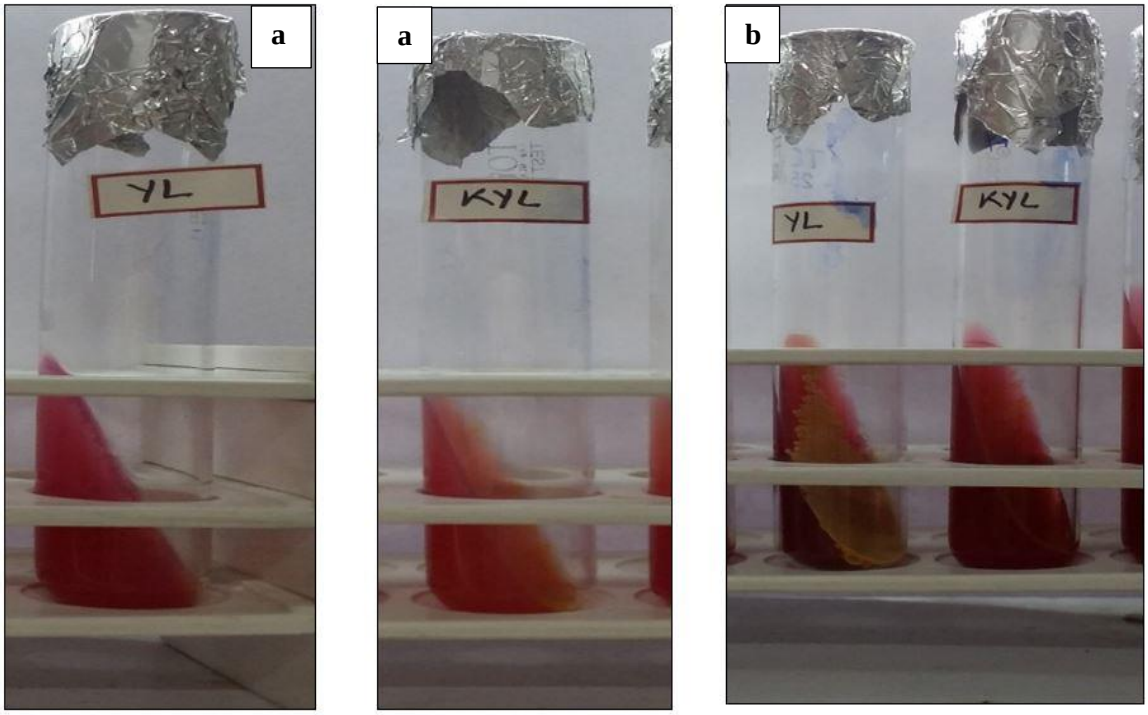


Fig. 3.4. (a)Tri Sugar Ion and (b) Urease test by *Pseudomonas punonensis* (KYL) and *Bacillus gibsonii* (YL)

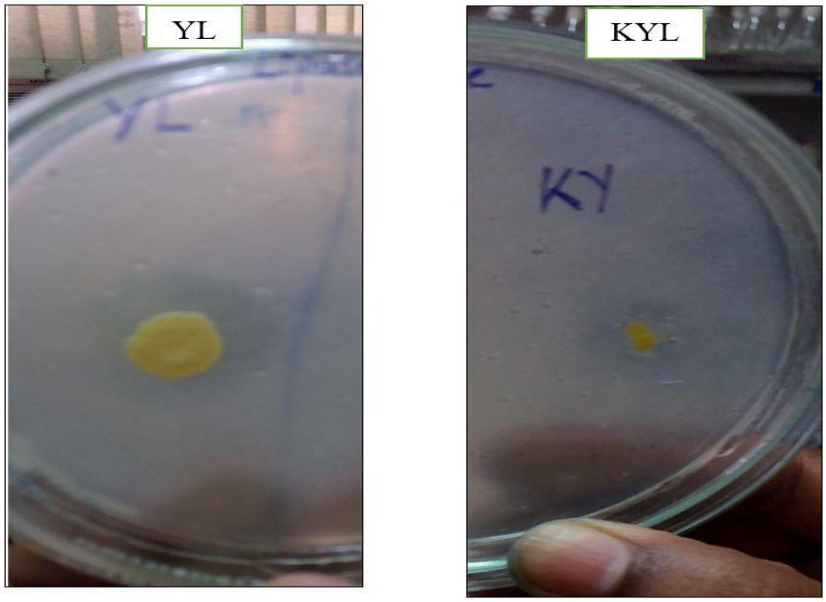


Fig. 3.5. Lipase production by *Pseudomonas punonensis* (KYL) and *Bacillus gibsonii* (YL)

E) Lipase test

Lipase producing microorganisms are screened by using Tributyrin (1%) in culture medium. The microorganism uses tributyrin as a carbon source and hydrolyze it. The hydrolysis of tributyrin leads for making of a clear halo which confirms the lipase production. In the present study both psychrotolerant and halotolerant strains exhibited production of extracellular lipase enzyme and a clear halo was formed on TBA plate. Both the strains produced zone of hydrolysis or clear halos around 3 to 6 mm size (fig. 3.5.). Veerapagu et al. (2013) also isolated a lipase producing *Bacillus* sp. on Tributryin agar plate. Wu et al. (2004) reported the kinetic of tributyrin hydrolysis by *P. fluorscenes* CCRC-(17015) in liquid –liquid and liquid –solid-liquid system in a batch reactor. Bharathi et al. (2019) isolated a *Pseudomonas* sp. from oil contaminated soil and screened its extracellular lipase property on TBA plates. Dahiya et al. (2010) also isolated *Pseudomonas mendocina* M-37 from soil which showed its extracellular lipase production when 1% olive oil was added as a carbon source in culture medium. Ertuğrul et al. (2007) isolated a lipase producing *Bacillus* sp. from olive mill wastewater by using tributyrin in culture medium. A lipase producing *Bacillus* sp. was isolated from soil and was found to be effective in the bio treatment of leather effluent (Kanimozhi et al., 2013).

Table: 3.2. Biochemical and Enzyme activity of *P. punonensis* and *B. gibsonii*

Biochemical and Enzyme test	***P. punonensis***	***B. gibsonii***
Amylase	-	-
Protease	-	-
Cellulase	-	-
Lipase	+	+
Urease	+	+
Triple sugar iron	+	-
Citrate utilization test	+	+

(+) symbolizing production of enzymes, (-) symbolizing no production

3.4 Conclusion

Two extremophilic bacterial strains were isolated from different extreme conditions. One psychrotolerant bacterial strain was isolated from agricultural soil of Leh, Laddakh (India) and other halotolerant was isolated from coastal saline water from Dwaraka, Gujrat, India. Tests revealed that the Gram negative psychrotolerant belongs to genera *Pseudomonas* and other strain is Gram negative halotolerant belonging to genera *Bacillus*. They have yellow or light-yellow colonies. The cells are rod shaped with rough edges. Based on the 16S rRNA gene sequencing and comparison with NCBI GenBank database, the psychrotolerant strain was identified as *Pseudomonas punonensis* (accession no. MH393314) and halotolerant *Bacillus gibsonii* (accession no. MK835660). Biochemical characteristics of *P. punonensis* and *B. gibsonii* revealed that both the strains could efficiently produce extracellular lipase and urease. The isolated strain showed positive result for citrate utilization and triple sugar iron.

CHAPTER 4

CHAPTER 4

KINETIC AND THERMODYNAMIC STUDY OF EXTRACELLULAR LIPASES ISOLATED FROM EXTREMOPHILIC BACTERIAL STRAINS

4.1. Introduction

Extremophiles are microorganism that have adapted to extreme environment. These extremophilic organisms fall into different classes that include psychrophiles, thermophiles, halophiles, acidophiles and many others. Psychrophiles are considered as extremophiles as they not only grow at low temperatures, but they modify their metabolic systems to face further environmental constraints. Psychrophilic microorganisms successfully colonize all permanently cold environments ranging from the deep sea to mountain and polar regions. Some of these organisms, depending on their optimal growth temperature, are also known by the terms psychrotolerant or psychrotrophs. Morita (1975) defined that the psychrophilic microorganisms that have optimum growth temperatures of <15°C and upper limit of cold tolerant up to of ~20°C. Psychrophilic organisms are further categorized, on the basis of growth temperature, into psychrophiles and psychrotrophs/psychrotolerants. The optimum growth temperature for the psychrophiles is below 15°C; however, for psychrotrophs it is about 20-25°C (Morita 1975). Such definition is unclear because of three main reasons, the first reason is its dependence on the temperature, which has been arbitrarily selected, and secondly, the definition does not apply on most of the eukaryotes. The last important reason is that microorganisms behave as thermodynamic units: increasing the ambient temperature increases the rate of metabolic reaction and growth rate.

Pseudoalteromonas, *Moraxella*, *Psychrobacter*, *Polaromonas*, *Psychroflexus*, *Polaribacter*, *Moritella* and *Vibrio* are represented as psychrophilic Gram-negative

bacteria. The category of Gram-positive bacteria includes *Arthrobacter*, *Bacillus*, *Micrococcus*, *Methanogenium*, *Methanococcoides* and *Halorubrum* species, belonging to group Archaea. Yeasts *Candida* and *Cryptococcus* species and fungi such as *Penicillium* and *Cladosporium* are found in the cold environments and display striking cold-adaptive characteristics (Feller et al., 2003).

The cold-adapted enzymes produced by psychrophilic microorganisms have flexible structures which are designed to compensate the low kinetic energy limitation present in cold environments. In general, low temperatures have an adverse effect on the physical properties and functions of membranes that may reduce the membrane fluidity. Chintalapati et al. (2004) and Russell, (1990) reported that low optimum temperatures produce a high amount of unsaturated, polyunsaturated and methyl-branched fatty acids, and/or a shorter acyl-chain length, high proportion of *cis*-unsaturated double-bonds and antesio-branched fatty acids. On comparison with mesophilic and thermophilic homologues, the cold-adapted enzymes show reduction in activation enthalpy and a more negative entropy due to their flexibility (Siddiqui and Cavicchioli, 2006). Cavicchioli et al. (2011) reported that temperature regulates the reaction rate of enzyme and with decrease in temperature, the cold-adapted enzyme reaction rate tends to decrease more slowly as compared with similar enzymes from thermophiles.

The thermolabile cold-adapted enzyme have some unique compositional and structural flexibility that is generally opposite to that of more rigid and stable mesophilic and thermophilic homologues (Siddiqui and Cavicchioli, 2006; Collins et al., 2008). Cold-adapted enzymes tend to possess various features, which confer stability in cold environment. The specific feature includes decreased core hydrophobicity, increased surface hydrophobicity, lower arginine/lysine ratio, and weaker inter-domain and inter-subunit interactions. The secondary structure of enzyme consists of longer loops, more

glycine residues, less proline residues in loops, more proline residues in α-helices, less and weaker metal-binding sites, a reduced number of disulfide bridges and fewer electrostatic interactions (H-bonds, salt-bridges, cation–pi interactions, aromatic–aromatic interactions). Genomic comparison of psychrophiles vs thermophiles has shown distinct biases in amino acid composition which is a trademark of thermal adaptation (Saunders et al., 2003). According to Treichel et al. (2010) and Tran et al. (2013), the lipases are water–soluble triacylglycerol (EC 3.1.1.3) that catalyze insoluble long chain triacylglycerides into fatty acids. Besides their natural function, they possess considerable potential to performed bioconversion reactions in non-aqueous media. Lipase can also hydrolyze organic carbonates in the absence of any cofactor (Pandey et al., 1999; Sharma et al., 2001; Dutra et al., 2008; Kakde et al., 2011). Lipases are serine class of hydrolase defined as triacylglycerol acylhydrolases which possess some unique properties such as chemioselectivity, regioselectivity and enatioselectivity (Stergiou et al., 2013). Besides, they also retain some unique features such as stability in organic solvents, low thermostability at high temperature with low cost and enantioselective properties (Thakur 2012; Vaseghi et al., 2012). The cold-active lipase has the capability to tolerate the extremely unfavourable condition of cold habitat with high bio catalytic activity. There is need to avoid any structural damage which confers irreversible adaptation to these environments and enables the enzyme to overcome the adverse influence of low temperatures compared to the lipases from mesophiles or thermophiles. To their functional and structural adaptation, the enzyme stability, activity, and flexibility are the most important factors (Margesin et al., 2007; Buzzini et al., 2012; Feller 2013). Psychrotolerant lipase are considered to be the best for economic application due to their low energy cost which is achieved by reducing the heating steps required to function in cold environments. To achieve an increased reaction yield, and minimizing undesirable reactions that occur

due to high temperatures induced heat inactivation of the enzymes, use of chemical extraction offers significant advantages to the enzyme by preventing any modification to the substrates and finished product.

Due to a massive variation in the applicability of lipases, there is a need to explore new enzymes to meet the demand of industries. Lipases can improve the washing capacity of the surfactants, which contain protease and easily remove the tough stain of fatty food and fabric sebum, which are hard to remove under normal washing conditions (Rathi et al., 2001). Ideally, alkaline lipases play an important role in detergent industry due to their high catalytic activity and stability over a wide pH range, which makes compatible with different constituents of detergent including metal ions, surfactants and oxidants (Wang et al., 1995). Several lipases with high activity at alkaline pH have been obtained from various microorganisms, such as *Achomobacter* sp. *Alcaligenes* sp. *Bacillus* sp. *Humicola* sp. *Pseudomonas* sp. (Lin et al., 1996). Khosla et al. (2017) have also reported alkaline lipase produced by *Pseudomonas sp*. ISTPL3 with optimum pH at pH 9.0. Mobarak et al. (2011) have also obtained an extracellular lipase from *P. aeruginosa* KM110 with an optimum pH of 9.0. This is the first report of extracellular alkaline lipase activity from *P. punonensis* with its maximum activity at alkaline pH 9.0. This novel enzyme has been studied to evaluate its lipolytic activity, optimization conditions, kinetic and thermodynamic parameters.

Halophile microorganisms can survive in saline environment due to their ability to maintain osmotic balance. They maintain a isotonic condition with environment either by absorbing sodium and potassium salts or by synthesis of compatible solute. Thus, the protein produced by halophilic microorganisms have the ability to cope up with high salt concentration (e.g. KCl concentrations of 4 M and NaCl concentrations of >5 M (Van Den Burg 2003). Larsen et al. (1986) described four categories of halophilic microorganisms based on their salt

tolerance ability. Those required ≤1 % NaCl for their growth and metabolism are considered non-halophilic; whereas, slightly halophilic and moderately halophilic are stimulated in the presence of 2–3 % and 5–10 % NaCl, respectively; and a higher than 10 % NaCl requirement indicates extreme halophilia. Li et al. (2014) has isolated *Idiomarina* sp. W33 from Yuncheng Salt Lake with unique characteristics of lipase. The lipase showed thermostability, alkaline and salt-tolerant features, which makes them more potent for industrial application. The protein of halotolerant bacteria adopts to extreme saline environment by acquiring a large number of negatively charge amino acid residue on their surface to prevent precipitation. However, with the low salt concentration the solubility of the halophilic enzyme became low which might restrict their wide industrial application (Madern et al., 2000). This property has been beneficial when halophilic enzymes are applied in aqueous and non-aqueous media.

Balaji et al. (2014) isolated *Bacillus* sp. VITL8 from oil-contaminated site that retained >55 % of lipase activity at alkaline pH with 60°C in 3 M NaCl concentration. The halotolerant lipase was stable with 25% (v/v) of acetonitrile and 20% of butanol. The hydrolytic specificity of lipase was found to be more towards long chain fatty acids such as olive oil and sunflower oil. The unique characteristics of lipase might be useful in various green technological process of industrial importance.

Thus, lipase produced from halotolerant microorganisms have some advantages, particularly with their thermal stability and organic solvent tolerance. Besides, to their halophilic characteristics, they are equally applicable in industrial process. In this study, the Psychrotolerant *P. punonensis* was isolated from agricultural soil of Leh, Laddakh (India) and halotolerant *B. gibsonii* was isolated from the water sample of Dwaraka, Gujrat (India). Both the isolated strains were checked for their morphological, biochemical and

molecular identification as mentioned in previous chapter. The kinetic and thermodynamic properties of lipase have been studied here.

4. 2. Materials and methods

4.2.1. Production of extracellular lipase

The isolated extremophilic bacterial strains were used for production of extracellular lipase in submerged culture process. The lipase production medium was prepared for each isolated strain in 250 mL Erlenmeyer flask containing 100 mL of medium (g/L): beef extract 1.5, yeast extract 1.5, peptone 5.0, NaCl 5.0, 1% olive oil with pH 7.2±0.2. However, the optimum salt concentration was used (600 mM) for the preparation of halotolerant lipase production media. The prepared culture medium was autoclaved for 15 min at 121°C by inoculating isolated extremophilic bacterial culture (2% v/v). The inoculated cultures were incubated at 30°C under shaking at 150 rpm for 72 hr. After incubation, the cultured broth was centrifuged at 12,000 rpm for 15 min at 4°C. The resulting cell free supernatant was used as a source of extracellular lipase enzyme stored at 4°C and subjected for partial purification and characterization**.**

4.2.2. Lipase assay and protein determination

The lipase activity was calculated by using p-nitrophenyl palmitate (p-NPP) as substrate (Ertugrul et al., 2007). The reaction mixture was freshly prepared by mixing solution A (30 mg of pNPP in 10mL of isopropanol) with solution B (0.1 g of gum Arabic and 0.4mL Triton X-100 in 90 mL 0f 50 mM Tris-HCl buffer, pH 8.0), by stirring the mixture until all the chemicals were dissolved. The final reaction mixture for enzyme assay was prepared by mixing 9 µL of substrate solution and 1µL of enzyme solution. The enzyme assay mixture was incubated at 25±0.1°C for 15 min and absorbance of the mixture was measured at 410 nm in a double beam UV-visible spectrophotometer. The molar extinction

coefficient (ε) of p-nitrophenol (pNP), ε=1.711 mM^{-1} under the condition was determined from the standard curve for pNP (Gomes et al., 2011). One unit of lipase activity was denoted as 1 μmol of p-nitrophenol released per minute under the assay conditions. Dissolved protein concentration was determined by the method of Lowry (Lowry et al.,1976), using bovine serum albumin (BSA) as a standard. The specific activity of enzyme was calculated as described by Massadeh et al. (2011).

4.2.3. Ammonium sulphate precipitation

The partial purification of lipase was performed at 4°C by using 0.2 M phosphate buffer (pH 7.0±0.2). The enzyme present in the supernatant was partially purified by salting out with ammonium sulphate at concentrations range from 40- 70% saturations. Subsequently, the solutions were centrifuged at 12,000 rpm for 15 min and the supernatant was discarded and remaining precipitate was collected. The precipitate was further dissolved in of 0.2M phosphate buffer (pH 7.0±0.2). After the ammonium sulphate precipitation, the excess ammonium salt in the solution was removed by dialyzing the protein by submerging dialysis tube in 0.2 M phosphate buffer pH 7.0. After every 1 hr the phosphate buffer was changed to obtained the appropriate purification. The partially purified enzyme was stored at -4°C and used for characterization (Toida et al., 1995).

4.2.4. Biochemical characterization of extracellular lipase

Biochemical, kinetic and thermodynamic properties of extracellular lipase from psychrotolerant *P. punonensis* and halotolerant *B. gibsonii* were studied further.

A) Effect of pH on lipase enzyme

The ideal pH for extracellular lipase was observed by incubating enzyme at 30°C under different pH conditions ranging from 4.0 to 11.0. The optimal enzyme activity was

determined by incubating the assay mixture enzyme under different pH conditions for 30 min. The different buffers as 50 mM sodium acetate buffer was used for pH 4.0–6.0, 50 mM phosphate buffer was used for pH 6.0–7.5, 50 mM Tris–HCl buffer for pH 8.0 -10.0 and 50mM Tris–glycine for pH 9.0 -11.0 was as described by Sinchaikul et al. (2001).

B) Effect of metal ions on lipase activity

Lipase activity was measured in the presence of different metal ions. The reaction mixture was pre-incubated with the different metal ions in 50 mM Tris buffer (pH 8.0) at 30ºC for 30 min. The metal solutions of $AlCl_3$, $ZnSO_4$, $BaCl_2$, $MgCl_2$, $MnSO_4$, $CaCl_2$, $CoCl_2$, KCl, NaCl (1 mM each) were added to the reaction mixture before measurement of lipase enzyme.

C) Effect of different inhibitors

The effect of different enzyme inhibitors on the lipase enzyme was recorded by pre-incubating the reaction mixture at pH 8.0 (50 mM Tris buffer) for 30 min. at 30°C. The enzyme inhibitors viz. dithiotheitol (DTT), 2-mercaptoehanol (BME), phenylmethylsulfonyl fluoride (PMSF), ethylene diamine tetra acetic acid (EDTA) were used at a final concentration of 1 mM, were added to the assay mixture before measurement of lipase activity. The assay mixture without addition of inhibitors served as control.

D) Effect of organic solvents and surfactants

The effect of different organic solvents on the lipase enzyme was studied by pre-incubating them at pH 8.0 (0.05 M Tris buffer) at 30ºC. The enzyme organic solvent viz. acetone, ethanol, methanol, butanol and isopropanol and different surfactants sodium dodecyl sulphate (SDS), Cetyl trimethyl ammonium bromide (CTAB), Tween-20, Tween-80 Triton X-100 were used at a final concentration of 1% (v/v) in the reaction mixture. The assay mixture without organic solvent was taken as control. After incubation for 30 min, enzyme assay was performed by using p-NPP substrate.

E) Effect of temperature on lipase activity

The reaction mixture of partially purified lipase enzyme (50 mM Tris-HCl, pH 8.0) was incubated for 30 min at different temperature ranging from 10°C to 70°C for psychrotolerant and 20°C -80°C for halotolerant strain to study the effect of temperature on lipase enzyme.

4.2.5. Kinetic and thermal behaviour of partially purified lipase

A) Kinetics and thermal properties of lipase

Enzyme activity was measured at different temperatures. The reaction rate constant (k) of enzyme at each temperature was calculated. The activation energy (E_a) was determined from the slope of the Arrhenius plot, which was plotted between logarithms of reaction rate constant versus reciprocal of absolute temperature. The kinetic values were calculated by the following formula (Prajapati et al., 2014).

$$k_{cat} = (k_b T / h) \times e^{(-\Delta H^* / RT)} \times e^{(\Delta S^* / R)} \quad (3.1)$$

k_{cat} = catalytic constant

Where, k_b = Boltzmann's constant (R/N) = 1.38 $\times 10^{-23}$ J/K

T = Absolute temperature (K)

h = Planck's constant = 6.626 $\times$ 10^{-34} Js

$R = 8.314 J / K / mol$ (gas constant)

ΔH^* = change in enthalpy

ΔS^* = change in entropy

Enthalpy (ΔH^*) of activation for each temperature was calculated by following equation

$$\Delta H^* = E_a - RT \quad (3.2)$$

The Gibb's free energy was calculated from the first- order rate constant:

(Gibb's free energy of activation) $\Delta G^* = -RT \ln(k_{cat} h / k_b T)$ (3.3)

From the equation (2) and (3), the activation entropy (ΔS^*) for extracellular lipase was calculated from the following equation:

$$\Delta S^* = (\Delta H^* - \Delta G^*) / T \quad (3.4)$$

B) Thermal inactivation of lipase enzyme

Enzyme inactivation can be carried out by different factors but the most important method of inactivation is thermal inactivation. The thermal inactivation kinetics was performed by pre-incubating the enzyme at various temperatures (60, 70 and 80°C) at pH 8.0 as described by Whitaker (1994). The residual activity of the enzyme after 30 min was determined in terms of percent residual activity obtained at room temperature as mentioned below: (Olusesan et al., 2011).

$$\text{Residual Activity (\%)} = \frac{C_t}{C_o} \times 100 \quad (3.5)$$

C_t = enzyme activity at time t (min)

C_o = enzyme activity at time 0 (min)

Enzyme inactivation often follow first-order kinetic model, which revealed that enzyme activity decreased log linearly as a function of time. The first-order inactivation rate constant was calculated by the following equation (Ortega et al., 2004).

$$\ln \frac{C_t}{C_0} = -k_d t \quad (3.6)$$

Where, C_t = enzyme activity at time t

C_0 = enzyme activity at 0 min

t = time of enzyme treatment

k_d = inactivation rate constant

Enzyme inactivation of lipase was determined by incubating the reaction mixture in 50 mM of Tris-HCl buffer from 15 to 120 min at different temperature as 30, 50 and 70°C for psychrotolerant and 60,70 and 80°C for halotolerant strain as described by Whitaker et al. (1994). An aliquot (1 mL) of enzyme was withdrawn from temperature treatment reaction mixture at regular time interval, cooled for 15 min and enzyme assayed was performed under standard condition. The deactivation rate constant (k_d) at each temperature was determined as mentioned below. (Prajapati et al., 2014);

$$k_d = (k_b T / h) \times e^{(-\Delta H^* / RT)} \times e^{(\Delta S^* / R)} \quad (3.7)$$

The deactivation energy ($E_{a(d)}$) for inactivation reaction was determined by the Arrhenius plot against ln (k_d) vs 1/T(K). The half – life ($t_{1/2}$) of enzyme was defined as the time at which the residual activity reached 50% which was calculated by the following expression:

$$t_{1/2} = \frac{\ln(2)}{K} \quad (\text{min}^{-1}) \quad (3.8)$$

Free energy change (ΔG^*) for inactivation of lipase was calculated by using following equation:

$$\Delta G^* = -RT \ln(k_d h / k_b T) \quad (3.9)$$

Decimal reduction time (D value) is defined as describe as the time required by enzyme to reduce 90% of initial activity at a specific temperature the D value was calculated as follows:

$$D = 2.303 / k_d \quad (3.10)$$

4.2.6. Circular Dichroism spectroscopy of the lipase

Temperature dependent CD spectra of partially purified lipase was recorded between 260 to 200 nm on a JASCO J-1500 spectrometer. For CD spectrum analysis, a path length of

1mm and scan rate of 100 nm/min with a minimum of 3 accumulations was used. Protein concentration of the sample was determined by Lowry method in 50mM sodium phosphate buffer, pH 7.2. Temperature of the protein solution was maintained at 30°C by JASCO peltier type temperature controller during spectra measurement (Batumalaie et al., 2018). Temperature dependent changes in enzyme secondary structure were obtained by heating enzyme at a constant rate of 1°C per minute from 30 to 90°C and measuring the change in ellipticity at 208 nm and 222 nm. Thermal stability of the protein was recorded by incubating the protein at 50 and 70°C for 30 min. as compare with control at 30°C.

Statistical analysis

All the experiments in the present investigation were performed in triplicates and the data presented are average values ± standard deviation of triplicates experiments.

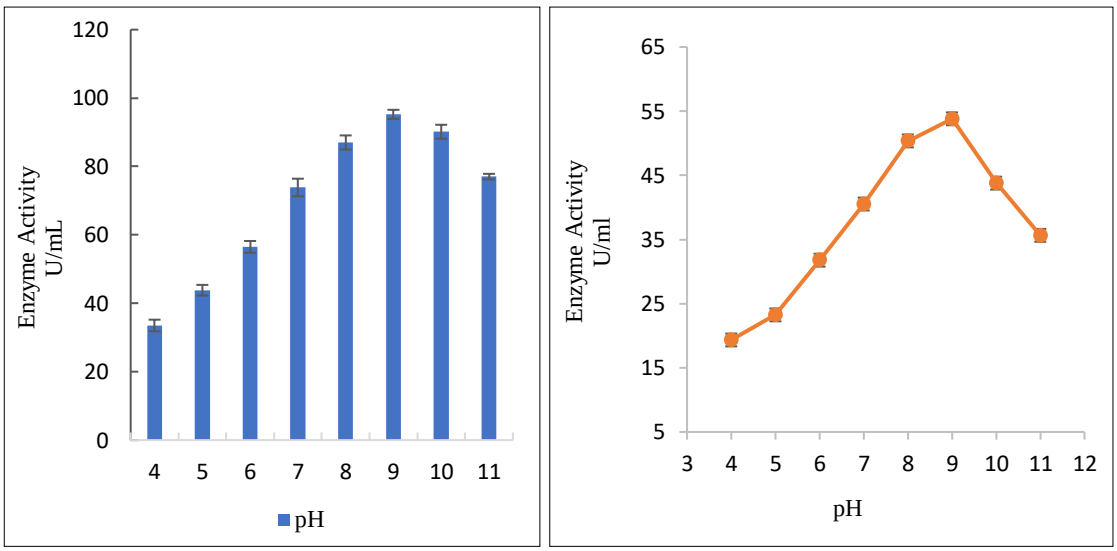
4.3. Results and discussion

4.3.1. Characterization of the extremophilic bacterial lipase

A) Effect of pH on lipase activity

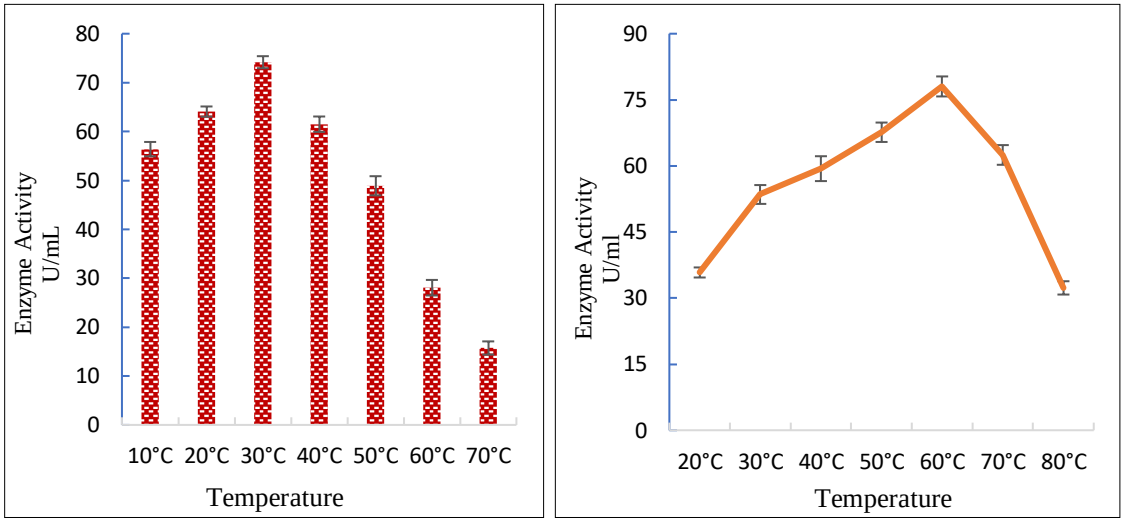
Lipase was incubated under different pH conditions (4.0-11.0) which revealed that maximum hydrolytic activity was detected between pH 9.0-11.0 for *P. punonensis* (Fig. 4.1.a) and pH 8.0 to 9.0 for *B. gibsonii* (Fig. 4.1.b), while in both cases enzyme activity was found to be low or below pH 7.0. In *P. punonensis* an increase in pH above 9.0 showed a decline in the enzyme activity. The optimum pH for lipase activity was observed at the alkaline pH 9.0. The result exhibited pH dependent increase in the extracellular enzyme activity from pH 4.0 to pH 11.0 (33.52 U mL^{-1} to 77.02 U mL^{-1}). Whereas, the optimum pH for extracellular lipase activity was found to be pH 9.0 (95.25 U mL^{1}). Gökbulut et al. (2013) reported that extracellular lipase from psychrotolerant *P. fluorescens* KE38 produced alkaline lipase with its maximum enzyme activity at pH 8.0.

Li and Zhang (2005) reported an optimum pH of 7.0-8.0 for thermostable lipase from *Geobacillus* sp. TW1, while Li and Yu (2013) reported that *Haloarcula* sp. G41 produced extracellular lipase with optimum activity pH 8.0. Tyndall et al. (2002) have also reported that the purified lipase form *B. stearothermophilus* P1 exhibited maximum activity at pH 8.5 and at 55°C temperature. These findings revealed that lipase was stable between the pH 8.0 to 9.0, but the enzyme activity registered decline at pH 10.0 and 11.0. However, the enzyme activity below pH 7.0, loses its activity (75 to 33%). In the previous report on *B. subtilis* NS8, the lipase activity was maximum at pH 8.0 (Olusesan et al., 2011).



(a) (b)

Fig 4.1 Effect of pH on lipase activity of (a) *P.punonensis* and (b) *B.gibsonii* .The data presented as mean ± S.D.



(a) (b)

Fig.4.2 Effect of temperature on (a) *P. punonensis* and (b) *B.gibsonii* lipase activity. The data presented are mean of three determinations and ± S.D.

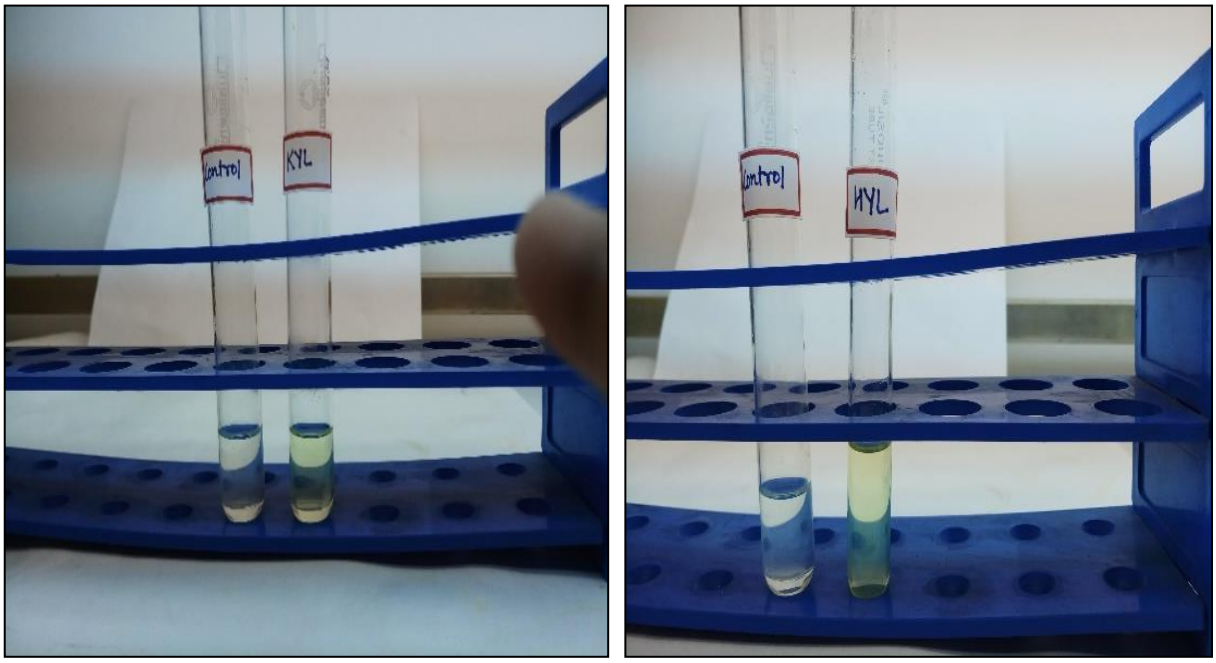


Fig. 4.2 c) Enzyme assay of extracellular lipase from *P. punonensis* (KYL) and *B. gibsonii* (YL)

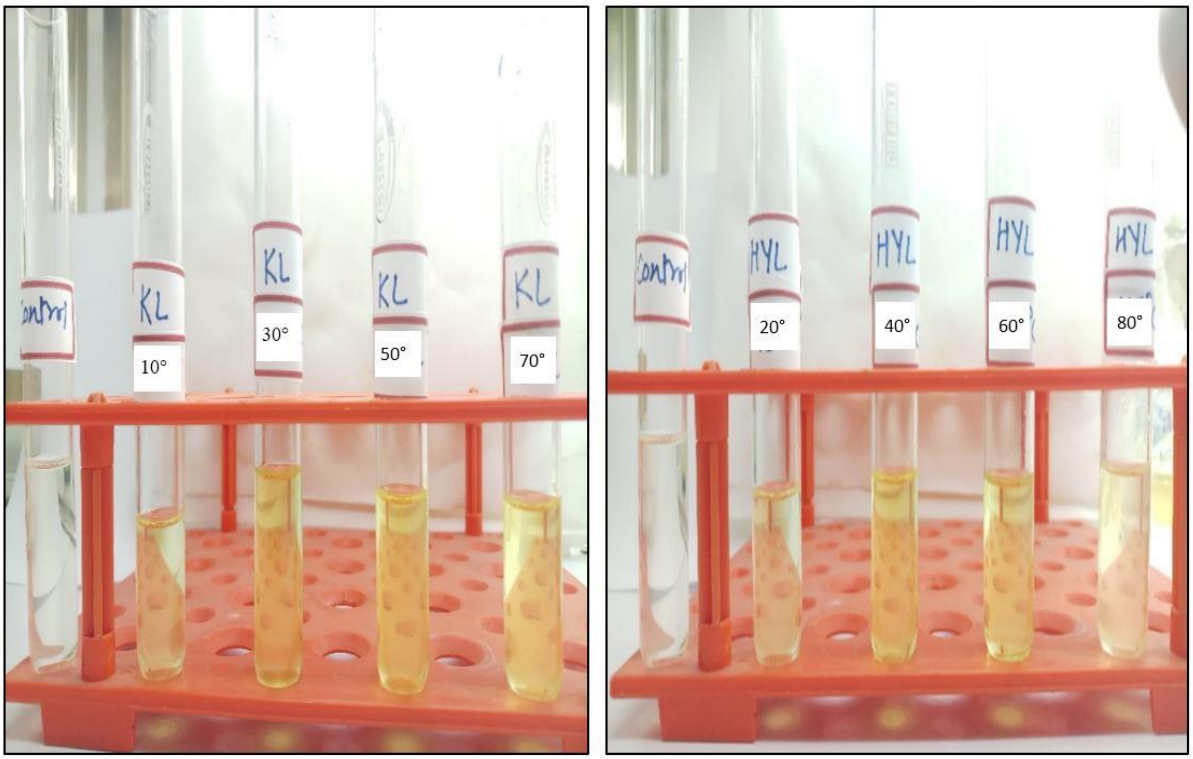


Fig. 4.2 d) Enzyme assay of extracellular lipase at different temperatures from *P. punonensis* (KYL) and *B. gibsonii* (YL)

B) Effect of temperature on extracellular lipase activity

The effect of temperature on lipase activity of *P. punonensis* and *B. gibsonii* was measured by incubating the enzyme at different temperatures (10°C to 70°C) and (20 to 80°C) respectively, at pH 8.0 for 30 min before addition of the substrate (Fig.4.2). The result of psychrotolerant strain (Fig 4.2.a) exhibited temperature dependent increase in the activity from 10°C to 30°C (56.4 U/mL to 74.17 U/mL). A further increase in the temperature from 30°C to 70°C showed substantial decrease in the hydrolytic activity (74.17 U/mL to 15.34 U/mL). Similar finding was reported by Salwoom et al. (2019) for purified lipase from psychrophilic *Pseudomonas* sp. LSK25. Similar findings were also observed in case of lipolytic strain of *Rhizopus oryzae* , which showed the highest lipase activity at 35°C

(Sharma et al.,2001). Rashid et al. (2001) reported that lipase enzyme from psychrotrophic *Pseudomonas sp*. strain KB700A which exhibited maximum activity between pH 8.0 and 8.5 with its optimum activity at 35°C.

The effect of temperature on *B. gibsonii* enzyme depicted in Fig 4.2.b, revealed a temperature dependent increase in the enzyme activity from 20°C to 60°C (78.03U/mL), which showed about 2-fold increase in the enzyme activity at 60°C temperature. A further increase in the temperature from 60 to 80°C led to a drastic decline in the hydrolytic activity of enzyme from 78.03 U/mL to 35.81 U/mL. Similar results were observed in *Geobacillus* sp., *Bacillus* sp., and *Geobacillus thermoleovorans* by Li et al. (2005), Nawani and Kaur (2000) and Abdel-Fattah and Gaballa (2008) respectively.

Table 4.1 a. Effect of different metal ions on lipase activity of *P. punonensis*

ADDITIVES	RELATIVE ENZYME ACTIVITY (%)
Control	100
CaCl2	138.88
BaCl2	115.13
MgCl2	118.54
KCl	111.71
AlCl3	121.99
MnSo4	85.40
ZnSo4	72.88
NaCl	112.23
CoCl2	72.44

Table 4.1 b. Effect of metal ions on extracellular lipase activity of *B. gibsonii*. The values presented as mean± S.D.

ADDITIVES	RELATIVE ENZYME ACTIVITY (%)
Control	100
CaCl2	106.3
BaCl2	103.29
MgCl2	82.87
AlCl3	153.19
MnSO4	57.99
ZnSO4	86.81
CoCl2	61.11

C) Effect of different metal ions on extremophilic lipase

The metal ions help in removing the fatty acids from the oil-water interface. Effect of different metals (1mM each) like Zn^{+2}, Ba^{+2}, Mg^{+2}, Mn^{+2}, Ca^{+2}, K^{+}, Al^{+3}, Na^{+}, Co^{+2} was studied on the lipase *P. punonensis* (Table 4.1 a) and *B. gibsonii* (Table 4.1 b). The results shown in Table 4.1 reveal that addition of Ca^{+2} stimulated the lipase activity (138.88%) of *P. punonensis* more than other metals as compared to control (without metals, 100%). Simultaneously, other metals such as $Ca^{+2,}$ Ba^{+2}, Mg^{+2}, Al^{+3}, K^{+}, Na^{+} also exhibited positive effect on enhancement of enzyme activity. However, the metals ions such as Zn^{+2}, Mn^{+2} and Co^{+2} showed inhibitory effect on the lipase activity. Similar result was reported by Makhzoum et al. (1995) in which Zn^{+2} and Co^{+2} inhibit the extracellular lipase activity of *P. fluorescens* 2D. In previous studies, extracellular lipase from *Bacillus methylotrophicus* PS3 was found to be stimulated by Mg^{+2} and Ca^{+2} ions (100 and 98.40% respectively), indicating the Ca^{+2} dependent increase in thermal stability of enzyme due to

change in the confirmation of enzyme protein (Sharma et al., 2017). Sharon et al. (1998) reported that Ca^{+2} enhanced the hydrolytic capability of extracellular lipase from *P. aeruginosa* KKA-5.

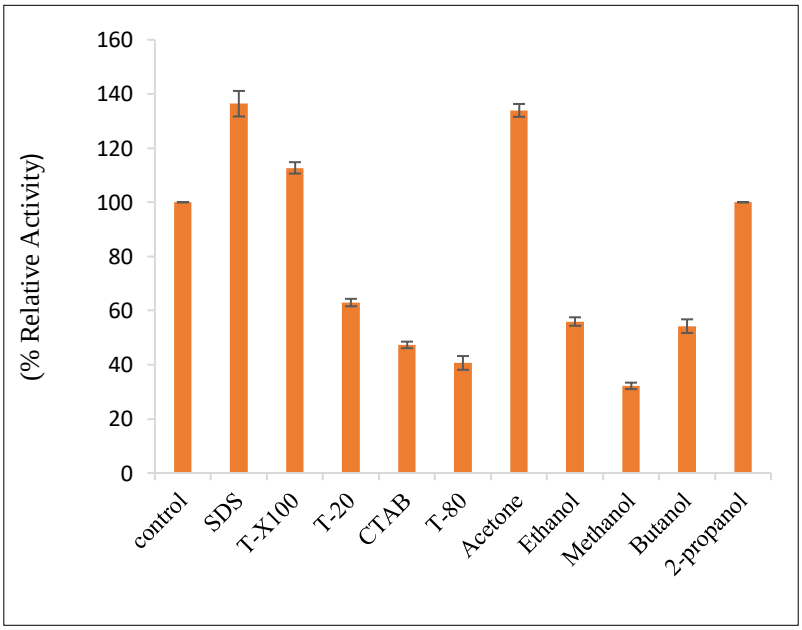
The effect of metal ions on *B. gibsonii* lipase activity (Table 4.1 b) showed that Mg^{+2}, Mn^{+2}, Zn^{+2} and Co^{+2} reduced the lipase activity (82.8, 57.9, 86.8 and 61.15%) as compared to control (100%). However, Ca^{+2} and Ba^{+2} did not show significant influence as the enzyme activity as compared to control. Surprisingly, the addition of Al^{+3} stimulated the lipase activity by 53.19% over that of control (100%). Sharon et al. (1998) reported that hydrolysis of oil was stimulated in the presence of some metal ions. Goswami and Basu (2012) observed that lipase activity was increased by 124% when the enzyme was treated with Al^{+3}. However, Ba^{+2} and Ca^{+2} showed minimal increase i.e. 103 and 106%, respectively. The extracellular lipase activity was uninhibited in the presence of Ca^{+2} and Ba^{+2} because they form salts of fatty acids which are insoluble and escape the inhibitory effect on product formation as suggested by Kambourova et al. (2003). In addition, Ca^{+2} confers the structural stability to enzymes against denaturation by heat and proteolysis. Rahman et al. (2005) revealed that metal ions binding to enzyme induces change in the conformational structure of enzyme which influences enzyme stability. In previous study, thermostable lipase from *B. stearothermophilus* P1 was slightly inhibited by Mg^{+2}, and Mn^{+2} (Sinchaikul et al., 2001).

D) Effect of different organic solvents, surfactants and inhibitors on lipase enzyme

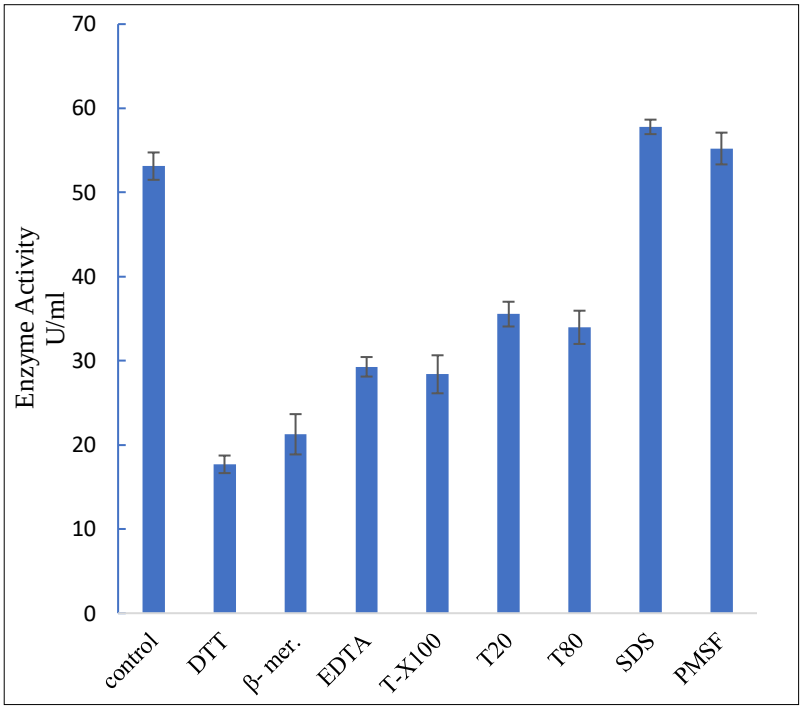
The organic solvents such as acetone, ethanol, methanol, butanol, iso-propanol, and surfactants such as Triton- X100, Tween 20, Tween 80, CTAB and sodium dodecyl sulfate (SDS) 1 mM each were taken to study their effect on the lipase activity of *P. punonensis* and *B. gibsonii* (Fig.4.3 a, b). The results showed that addition of Tween 20, Tween 80 and CTAB, inhibited the lipase activity by 62.96, 40.66 and 47.32%, respectively as compared

to control. Whereas, anionic surfactant SDS and non-ionic surfactant Triton X- 100 increased the enzyme activity 136.38 and 112.69% respectively. Silva et al. (2005) suggested that various ionic and non-ionic surfactants increase the cell permeability of microorganisms, which ultimately facilitate the transfer of numerous molecules across the cell membrane as well as increase protein secretion, by facilitating the contact between enzyme and substrate. Moh'd et al. (2010) reported that surfactant SDS bind to the active site of lipase enzyme and induce in the conformational changes in the enzyme, which facilitates the accessibility of enzyme to the substrate. These results were confirmed with the finding of Kavitha and Shanthi (2017) who observed that SDS enhanced the lipase activity due to action of surface-active agents which stimulate the interaction between the active site of the enzyme and the substrate by dropping the surface tension of the aqueous medium. All the organic solvents showed inhibitory effect on enzyme activity except acetone and 2- propanol, which stimulated the enzyme activity. The acetone indeed increases in the activity 133.91% as compared to control (100%) indicated that it was the best solvent for extraction of lipase. Karadzic et al. (2006) suggested that many organic solvents (10-20%) have adverse effects on lipase activity. Bisht et al. (2013) stated that the hydrophilic organic solvents exhibit more inhibitory effect on the lipase activity as compared to hydrophobic solvents. The result showed that addition of dithiothreitol (DTT)- and β-mercaptoethanol (BME) thiol group inhibitors inhibited the enzyme activity by approximately 62 and 71%, respectively, as compared to control (without inhibitor). However, addition of serine group specific protein inhibitor (PMSF) and EDTA – a known metal chelator stimulated lipase activity by 172 and 135%, respectively, when compared with control (100%). The enzyme activity was enhanced by S-H group inhibitor (PMSF), which suggests that thiol group played an important role in the enzyme activity (Jinwal et al., 2003).

The result of *B. gibsonii* (Fig.4.3b) showed that extracellular microbial lipase was sensitive to chelating agent EDTA, which inhibited the lipase activity by 55%, whereas dithiothreitol (DTT) strongly inhibited the enzyme activity by 33%. Thiol group inhibitor such as 2-mercaptoethanol also inhibited lipase activity by 40%. However, the serine group specific inhibitor PMSF and anionic surfactant SDS worked as an inducer for the enzyme activity. Both PMSF and SDS stimulated the extracellular lipase activity by 104 and 105 % over the control (100%). However, other non-ionic surfactants Triton X 100, T-20 and T-80 strongly inhibited the lipase activity by 32, 33 and 37 % as compared to control respectively. Royter et al. (2009) reported that extracellular lipase from *Thermoanaerobacter thermohydrosulfuricus SOL1* showed 76% inhibition by EDTA, while 11, 12 and 22% inhibition by Triton X 100, T-20 and T-80 respectively.



(a)



(b)

Fig 4.3 Effect of surfactants and organic solvents (1%) on lipase activity of represent (a) *P.punonensis* and (b) *B. gibsonii* (b). Data are means of three determinations and bars represents standard deviation

4.3.2. Kinetic parameters of extracellular lipase

The kinetic parameters of extracellular lipase enzyme from *P. punonensis* (Fig.4.4 a) and *B. gibsonii* (Fig.4.4 b) was studied by using pNP-palmitate (0.1- 1.5mM) as substrate under different temperatures (10, 30, 60°C). The Michaelis- Menten saturation kinetics showed initial concentration dependent increase in the rate of pNP-palmitate hydrolysis, which exhibited rate maxima at 1.0 mM concentration under all the temperature conditions. A further increase in the substrate concentration beyond reaction rate saturating substrate concentration (1.0 mM), the rate of pNPP hydrolysis remained unaffected, irrespective of temperature conditions (10, 30 and 60°C).

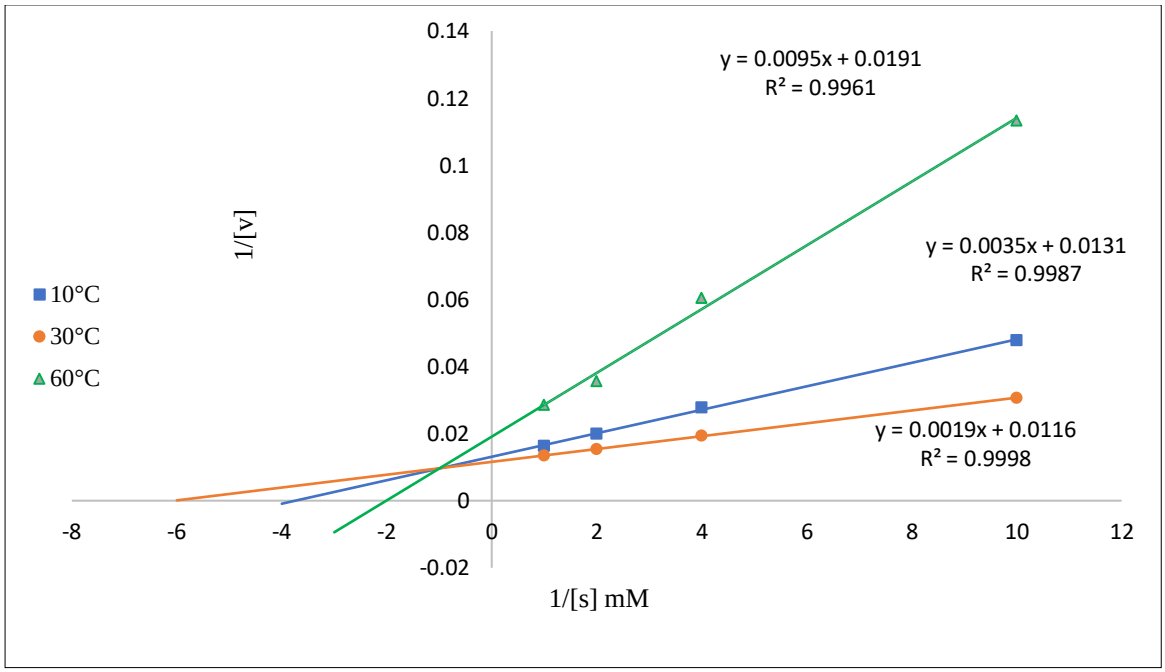
A reciprocal Lineweaver-Burk plot (Fig. 4.4 a) of the data derived from the Michaelis – Menten saturation kinetics were used for calculating the kinetic constants such as K_m, V_{max},

and $k_{cat.}$. The K_m values calculated for the enzyme activity at 10, 30 and 60°C were found to be 0.267, 0.163 and 0.479 mM respectively, for *P. punonensis* lipase. The V_{max} values of lipase enzyme at 10, 30 and 60°C were found to be 76.33, 86.22 and 52.08 U/mL respectively. As evident from the K_m and V_{max} values, the highest rate of enzyme activity (V_{max}) accompanied by higher affinity (lower value of K_m) of the enzyme towards substrate were observed at 30°C (Table 4.2 a). It was observed that change in temperature condition of enzyme led to drastic change in kinetic constants (K_m and V_{max}) and turn over number (k_{cat}). The enzymatic reaction rate (k_{cat}) is defined as the turnover number analogous to maximum number of substrate molecules transformed into product per active site per unit time. As the k_{cat} values calculated under all the temperature conditions were as 187.08, 208.76 and 127.02 min^{-1} at 10, 30, 60°C, respectively. The k_{cat} (turn over number) value for enzyme was maximum at 30°C, indicating higher rate of lipase activity. The catalytic efficiency constant (k_{cat} /K_m) at 30°C was found to be the best (1304.75), denoting the highest frequency of enzyme-substrate encounter at 30°C.

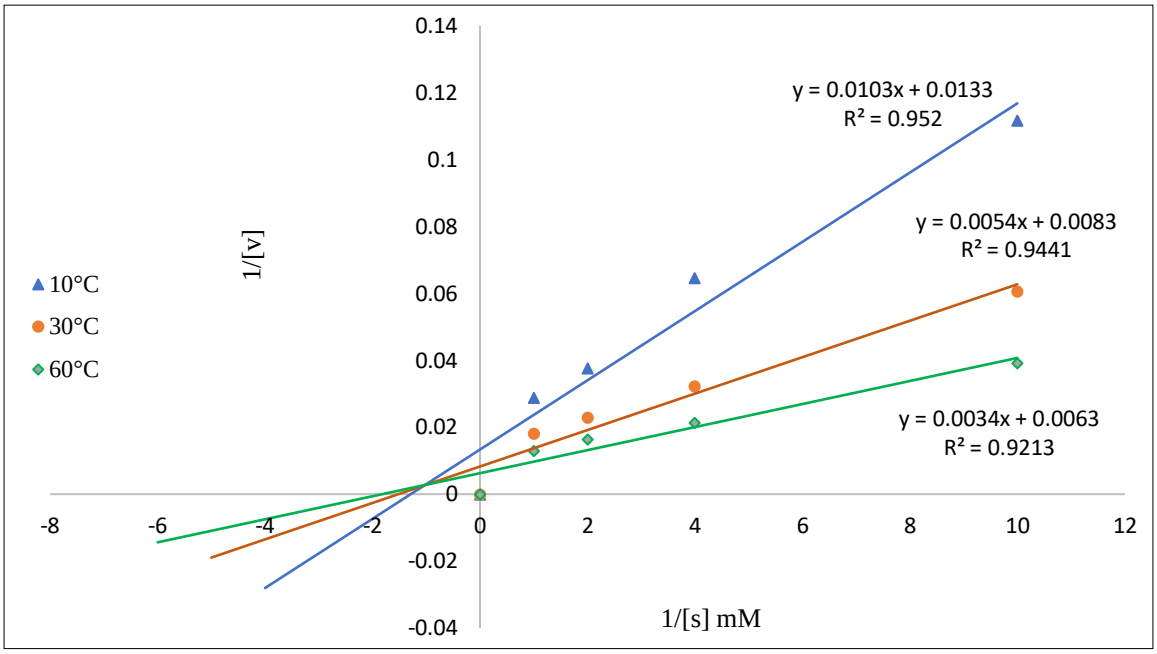
The kinetic study of extracellular lipase enzyme from *B. gibsonii* was carried out using varying concentration of pNPP (0.1- 1.5mM) as substrate under different temperatures (10, 30, 60°C). The result shown in Table 4.2 b, revealed that K_m values calculated for the enzyme activity at 10, 30 and 60°C were 0.77, 0.65 and 0.53 mM, respectively. The V_{max} values of lipase enzyme at 10, 30 and 60°C were 75.18, 120.48 and 158.73 U/mL, respectively. As evident from the K_m and V_{max} values of the enzyme, the highest rate of enzyme activity accompanied by higher affinity (lower value of K_m) of the enzyme towards substrate was observed at 60°C. A decrease or increase in the temperature led to a change in the K_m and V_{max} values. The k_{cat} (turn over number) values calculated under all the temperature conditions (10, 30, 60°C) showed that the highest k_{cat} value (483.93 min^{-1}) for

enzyme at 60°C, indicating higher turnover of lipase enzyme at this temperature. The catalytic efficiency constant (k_{cat}/K_m) at 60°C was found to be the best (913.07), denoting the highest frequency of enzyme-substrate encounter at 60°C.

The activation energy (E_a) of enzyme is the minimum amount of energy which is required to start an enzyme-substrate reaction. The lower activation energy denotes the faster rate of reaction. The activation energy (E_a) of enzyme, calculated from Arrhenius plot of the activity (Fig 4.5a), was 27.74 kJ/mol for extracellular lipase of *P. punonensis*, whereas it was 45.28 kJ/mol for lipase enzyme from *B. gibsonii* (Fig 4.5 b). These kinetic constants of enzyme suggested that the enzyme from psychrotolerant strain showed maximum efficiency at 30°C, whereas lipase from *B. gibsonii* was stable and exhibited its best activity at 60°C. A change in the temperature conditions of enzyme beyond their respective temperature optima creates a kinetic and thermodynamic barrier for the enzyme.

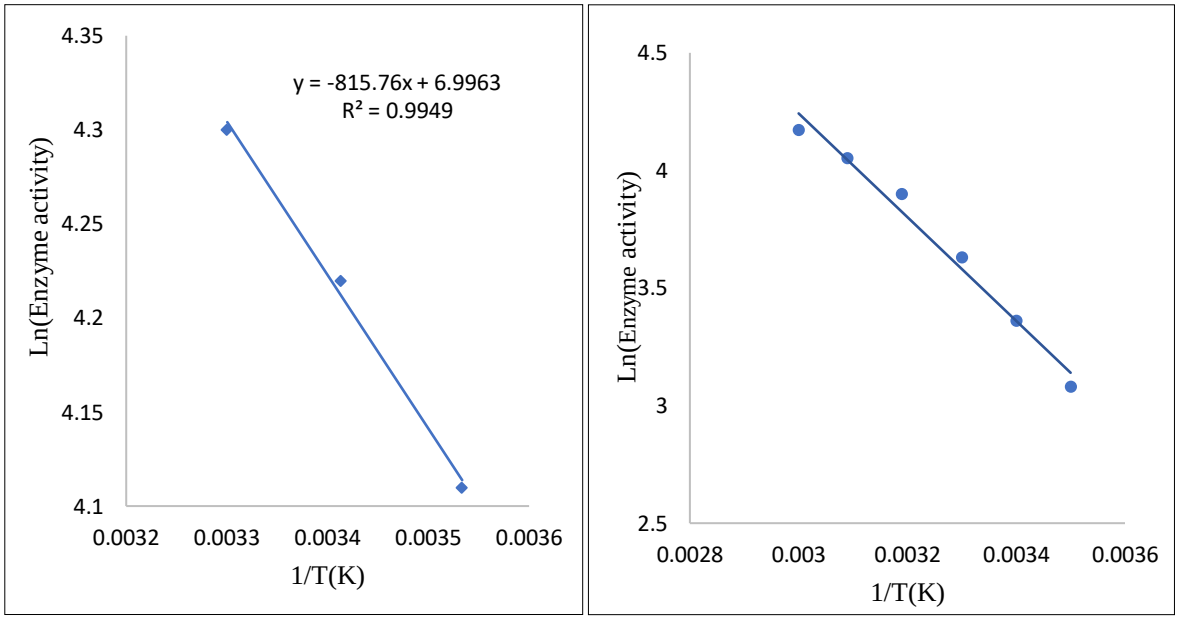


(a)



(b)

Fig 4.4 Lineweaver-Burk plot of (a) *P. punonensis* and (b) *B. gibsonii* (b) lipase using pNP-Palmitate as substrate over the substrate concentration range 0.1–1.0 mM at different temperature (10, 30 and 60°C) under standard assay conditions.



(a) **(b)**

Fig.4.5 (a,b) Determination of the activation energy of (a) *P. punonensis* and (b) *B. gibsonii* extracellular lipase based on Arrhenius plot.

Table 4.2 a. Kinetic and thermodynamic parameters of *P. punonensis* lipase for pNPP hydrolysis at different temperature

Temperature (°K)	K_m (mM)	V_{max} (U/ml)	k_{cat} (min^{-1})	k_{cat}/K_m
283	0.26	76.33	187.08	719.53
303	0.16	86.22	208.76	1304.75
333	0.47	52.08	127.02	270.25

Table 4.2 b. Kinetic and thermodynamic parameters of *B. gibsonii* lipase for pNPP hydrolysis at different temperatures

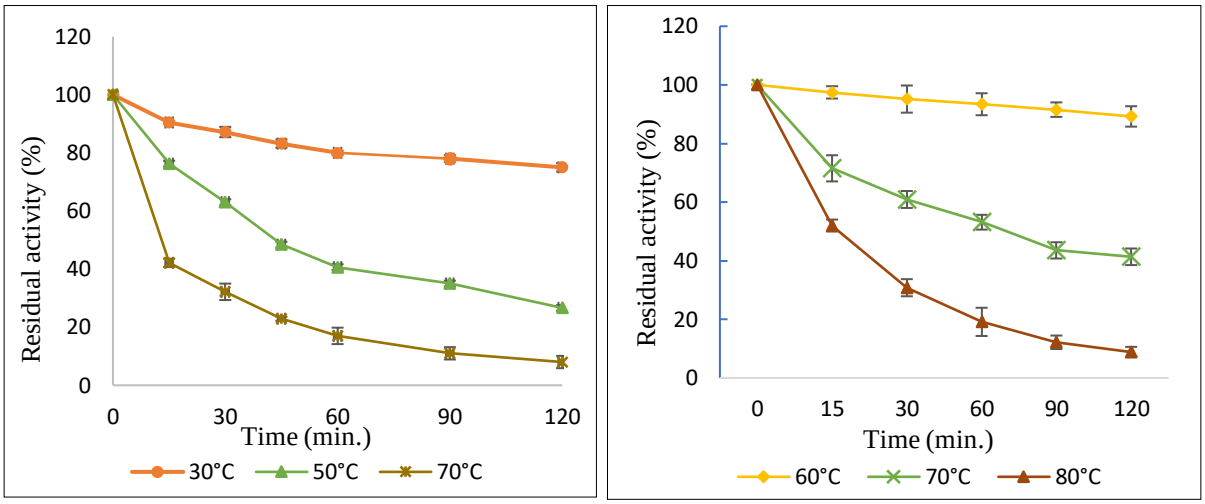
Temperature (°K)	K_m (mM)	V_{max} (U/ml)	k_{cat} (min^{-1})	k_{cat}/K_m
283	0.77	75.18	236.41	307.02
303	0.65	120.48	363.98	559.96
333	0.53	158.72	483.93	913.07

4.3.3. Thermodynamic characteristics of lipase

The thermal stability of psychrotolerant and halotolerant lipase was examined in terms of percentage residual activity of the enzyme (Fig 4.6 a) incubated at different temperatures for *P. punonensis* (30, 50 and 70°C) and *B. gibsonii* (60, 70 and 80°C). The initial enzyme activity at the start temperature treatment (0 min) was taken as control (100%, untreated). During the first 15 min of heat treatment of *P. punonensis* lipase at 30, 50 and 70°C, the enzyme exhibited 92, 72 and 53% residual activity respectively, as compared to control (100%) (Fig 4.6 a). After 30 min of enzyme incubation at 30, 50 and 70°C, the residual enzyme activity declined to 87, 63 and 32% respectively. However, the residual enzyme activity after 90 min treatment at different temperatures (30, 50 and 70°C) was reduced to 78, 37 and 16% respectively, when compared with their respective untreated control

(100%). After long-term (120 min) temperature treatment of enzyme at various temperatures (30, 50 and 70°C), the residual enzyme activity was found to be 75, 26 and 10% respectively. These results suggested that thermal denaturation of the *P. punonensis* enzyme was not only dependent on temperature intensity, but duration of temperature treatment was also an important factor. Similar findings were observed in extracellular lipase from *Cellulomonas flavigena* UNP3 (Prajapati et al., 2014).

The thermal stability of lipase enzyme from halotolerant strain *B. gibsonii* was determined by incubating the enzyme at different temperature (60, 70 and 80°C). During the first 15 min of incubation at 60, 70 and 80°C the enzyme (Fig 4.6 b), the residual activity of enzyme was 97, 71 and 52%, respectively as compared to initial activity at 0 min. After 30 min of temperature treatment, the residual activity was 95, 60 and 30 % at 60, 70 and 80°C reactively as compared to control (100%). After 90 min of incubation of enzyme at various temperature (60,70 and 80°C) the residual activity declined to 89, 50 and 24% with respect to control (100%). The enzyme was able to retain 88% of residual activity after 120 min at 60°C, while at 70 and 80°C, the residual activity decreased to 41 and 10 %, respectively. Olusesan et al. (2011) and Kim (1994) reported that extracellular lipase from *Bacillus thermoleovorans* ID-1A and *Bacillus* strain at 60°C could retain 75% and 50% of its activity respectively, after 30 min treatment. The thermostable lipase of *G. thermodenitrificans* IBRL-nra was able to retain 87.5% of its initial enzyme activity after 15 min of temperature treatment at 70°C (Balan et al., 2012).



(a) **(b)**

Fig.4.6 (a, b) First-order kinetic plot for irreversible thermal denaturation of lipase from (a) *P. punonensis* and (b) *B. gibsonii* . The data presented as mean ± S.D. values

The results on the thermal inactivation of lipase enzyme (Fig 4.6 a) showed an increase with increasing temperature (30-70°C). The thermal inactivation rate constant k_d value increased from 0.005 min^{-1} at 30°C to 0.0611 min^{-1} at 70°C (Table 4.3a). It has been reported by Whitaker et al. (1994) that the inactivation rate constant (k_d) is always inversely proportional to the stability of the enzyme. Therefore, the lowest k_d value obtained at 30°C further justified that the lipase from *P. punonensis* was more stable at 30°C. Enzyme inactivation is generally defined a process which involved protein aggregation, dissociation or denaturation of protein, leading to conformational changes in the enzyme structure (Polakovic et al., 1998). The thermal inactivation energy ($E_{a(d)}$) constant, indicating an amount of minimum energy required to start the enzyme denaturation, was calculated from the slope of the plot of ln k_d versus 1/T (temperature) (Fig.4.7a). The thermal inactivation energy ($E_{a(d)}$) for lipase from *P. punonensis* was calculated as 56.89 kJ/mol by Arrhenius plot.

The thermal inactivation rate constant (k_d) for the lipase activity was calculated by linear regression of the slope obtained by percent remaining activity against time (min.). The

result presented (Fig 4.6 b) on lipase from *B. gibsonii* followed a first -order reaction kinetic model. The steady inactivation of lipase enzyme occurred with increase in temperature (60-80°C). This also showed increase in the kd value from 0.09 min^{-1} at 60°C to 0.182 min^{-1} at 80°C (Table 4.3 b). Whitaker (1994) reported that the inactivation rate constant (k_d) is inversely proportional to the stability of the enzyme. Therefore, the lowest k_d value obtained at 60°C further justified that *B. gibsonii* lipase was a thermostable enzyme.

The thermal inactivation energy ($E_{a(d)}$) was calculated as 66.98 kJ/mol, from the slope of the plot of ln k_d versus 1/T (temperature) (Fig.4.7b). The higher inactivation energy ($E_{a(d)}$) indicated that the enzyme inactivation needed higher thermal energy to change the enzyme structure (Segal, 1976). Therefore, thermal inactivation value for lipase from *B. gibsonii* denoted greater thermostability of enzyme. Kumari et al. (2012) reported that inactivation energy of thermostable lipase LIP8, LIP14 and LIP18, from *Yarrowia lipolytica* as 64.6, 56.1 and 66.5 kJ/mol respectively. Olusesan et al. (2011) investigated that the thermal inactivation energy for lipase from *B. subtilis* NS 8 was 76.0 kJ/mol.

Hydrophobic interactions are considered to play primary role in the stabilization of the native conformation of the proteins, but not in the unfolded protein (Kristjansson et al., 1991). Thermostable enzyme has capacity to retain their active structural conformation at high temperature due to major forces like hydrophobic effects and hydrogen bonding which are responsible for maintaining an active conformation. Thus, the overall balance is known as the free energy change, ΔG^*, of a protein (Pace, 1975). During the thermal treatment of enzyme, the ability of enzyme stabilization can be determined by change in Gibbs free energy (ΔG^*). The value of ΔG^* for *P. punonensis* lipase was calculated as 86.31 kJ/mol at 60°C and 91.89 kJ/mol at 80°C shown in Table 4.3 a. The observed Gibbs free energy value exhibited the significance of enzyme stability. The closely related result was reported by Owusu et al. (1992) in *P. fluorescens* P38 lipase, where Gibbs free energy was obtained

as 103-105 kJ/mol at 60-80°C. Adams et al. (1981) also reported Gibb' free energy (ΔG^{*}) as 112-121 kJ/mol for heat-resistance *Pseudomonas* sp. The values of Gibb's free energy ($\Delta G^{*)}$ for lipase from *B. gibsonii* was calculated as 87.44 at 30°C, which increased to 95.40 kJ/mol at 70°C (Table 4.3 b). Similar observation was made by Oulsesan et al. (2011) in case of extracellular lipase from *B. subtilis* N8.

The enthalpy (ΔH^{*}) is the amount of heat released or absorbed during an enzyme-substrate reaction occur at a constant pressure, and it denotes that it is the amount of energy which must be overcome by the reacting molecule. The value of enthalpy (ΔH^{*}) for lipase enzyme in *P. punonensis* was slightly influenced (54.29 to 53.95 kJ/mol) between 30-70°C (Table 4.3 a). The results suggest that the thermal denaturation of enzyme was more at high temperature. Owusu et al. (1992) reported the enthalpy of *Pseudomonas* P38 lipase was 170 kJ/mol, which was much higher than the value of ΔH for lipase from *P. punonensis*. The value of enthalpy was not high because of high rate of protein refolding at low temperature. The value of enthalpy for *B. gibsonii* are calculated as 64.26 to 64.04 kJ/mol for 60 to 80°C (Table 4.3 b). Similar results were observed for lipase from *Cellulomas flavigena* UNP3 sp. where enthalpy was reported as 59.56 -59.47 kJ/mol at 60-70°C, which was lower than ΔH value for lipase from *B. gibsonii* at the same temperature (Prajapati et al., 2014).

Entropy is the change in protein structure during folding of protein. Enthalpy and entropy both the parameters showed a direct relationship in enzyme stability. Both the parameters provide a significant relationship between the non-covalent bonds and net enzyme/substrate changes with the stability of enzyme (Ortega et al., 2004). Entropy (ΔS) values were obtained as -0.109, -0.121 and -0.120 kJ/mol/K at 30, 50 and 70°C, respectively (Table 4.3 a). Thus, negative entropy obtained for lipase of *P. punonensis* proved that the enzyme was

more stable at 30°C. Fox and Stepaniak et al. (1983), reported entropy of different lipases from *Pseudomonas* strains were -0.079 to 0.114 kJ/mol/K, which was similar to the value obtained for lipase from *P. punonensis.* Positive value of enthalpy is related with increased enzyme stability, but a large increase in the value of entropy promotes destabilization of protein. The result shown in Table 4.3a indicates the enzyme from *P. punonensis* was more stable at 30°C.

The entropy values obtained as -66.21×10^{-3} kJ/mol/K to -78.8×10^{-3} kJ/mol/K for 60-80°C respectively for lipase from *B. gibsonii* (Table 4.3 b). It is considered that the enzyme stability is induced with a moderately high level of (ΔH^{*}) and low level of (ΔS^{*}). The values of enthalpy and entropy obtained for lipase from *B. gibsonii* proved that the enzyme was more stable at 60°C. The entropy values for lipase from *B. subtilis* NS8 was -83.45 kJ/mol/K to -81.84 kJ/mol/K at temperature 60-80°C. A negative value of entropy for irreversible thermal inactivation of *B. gibsonii* indicated that at the high temperature, the enzyme molecules probably retain the unfolded conformational of enzyme (Adams et al., 1981).

The half-life of thermal inactivation of enzyme was taken as another important parameter to predict the thermostable nature of enzyme under different temperature regimes. The half –life of lipase enzyme from *P. punonensis* was calculated as 138.2 min. at 30°C, which was reduced to 43.59 min. at 50°C and 11.34 minute at 70°C. The half –lives of *B. gibsonii* extracellular lipase was calculated as 76.40, 58.23 and 38.07 min at 60, 70 and 80°C respectively. Sharma et al. (2002) and Kambourova et al. (2003) reported half -lives of thermostable lipase from *Bacillus* sp.RSJ-1 and *B. stearothermophilus* MC 7 as 150 min (60°C) and 180 min (70°C) respectively. Kim (1994) and Kim (1998) reported the half-

lives of lipase from *Bacillus* sp. strain 398 and *B. stearothermophilus* was 30 min at 65 and 62°C, respectively.

However, the D value of enzyme the time required to reduce the activity by 1^{10} fold, also revealed gradual decline in the values (460.6, 144.84 and 37.69 min^{-1}) under different temperatures (30, 50 and 70°C, respectively) for *P. punonensis* whereas, it was found to be as 253.91, 193.52 and 126.53 min^{-1} at 60, 70 and 80°C, respectively. The lowest D value indicated the maximum denaturation of the lipase enzyme. These results suggested that the inactivation of enzyme was spontaneous and the thermal stability of the enzyme declined with increase in the temperature. These findings indicated that thermostability of lipase enzyme from *P. punonensis* was maximum at 30°C and lipase from *B. gibsonii* showed maximum thermal stability at 60°C.

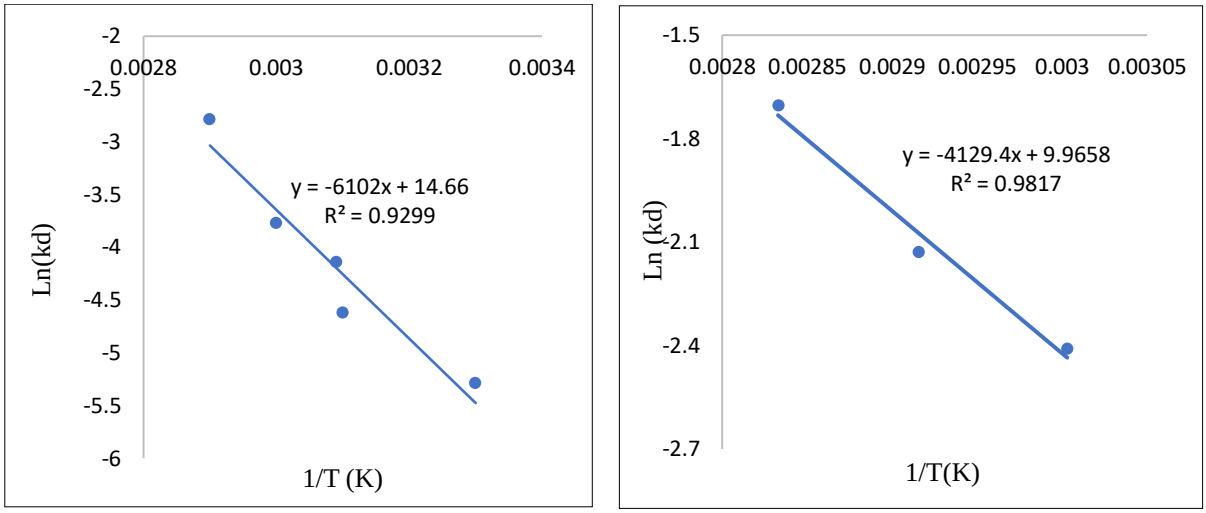
Table: 4.3 (a, b) Thermodynamic and kinetic parameters of irreversible thermal inactivation of lipase from (a) *P. punonensis* and (b) *B. gibsonii*

(a) *P. punonensis*

S.No.	Temperature (°K)	k_d (min^{-1})	D value (min)	$t_{1/2}$ (min.)	ΔH^* (kJ/mol)	ΔS^* (kJ/mol/K)	ΔG^* (kJ/mol)
1	303	0.005	460.6	138.2	54.29	-0.109	87.44
2	323	0.0159	144.84	43.59	54.11	-0.121	93.29
3	343	0.0611	37.69	11.34	53.95	-0.126	95.40

(b) *B. gibsonii*

S.No.	Temperature (°K)	k_d (min^{-1})	D (min)	$t_{1/2}$ (min)	ΔH^* kJ/mol	$\Delta S^* \times 10^{-3}$ J/mol/K	ΔG^* kJ/mol
1	333	0.0907	253.91	76.40	64.26	-66.21	86.31
2	343	0.119	193.52	58.23	64.13	-72.33	88.94
3	353	0.182	126.53	38.07	64.05	-78.8	91.89



(a) **(b)**

Fig.4.7 (a,b) Arrhenius plot of Ln (k_d) vs 1/T to determine the thermal denaturation energy of (a) *P. punonensis* lipase and (b) *B. gibsonii*

4.3.4. Secondary structure determination of lipase

CD spectra of the both isolated bacterial lipases were recorded at different temperatures (30, 50, 70 and 90°C) to assess the temperature dependent changes in the secondary structure of lipase enzyme. The far-UV CD spectra of lipase at room temperature (30°C) exhibited one negative peak at 210 nm (Fig.4.8 a, b), suggesting that lipase retained its native structure as observed by previously (Manan et al., 2018). The negative peaks at 210 indicate α-helix of secondary structure of protein. The intensity of negative peaks at 210 nm initially increased with rise in temperature up to 50°C in *P. punonensis* and 70°C *B. gibsonii*. Subsequent rise in the temperature from 70 to 90°C showed decline as well as shift in both the peaks, indicating deformation in the α-helix of the protein structure in both the strains. However, the changes in the peak intensity of α-helix structure was maximum at 90°C. The overall results of CD spectra revealed ordered structure of the enzyme at 30°C for *P. punonensis* lipase and 60°C for *B. gibsonii* lipase, followed by high temperature induced gradual deformation in the secondary structure of protein.

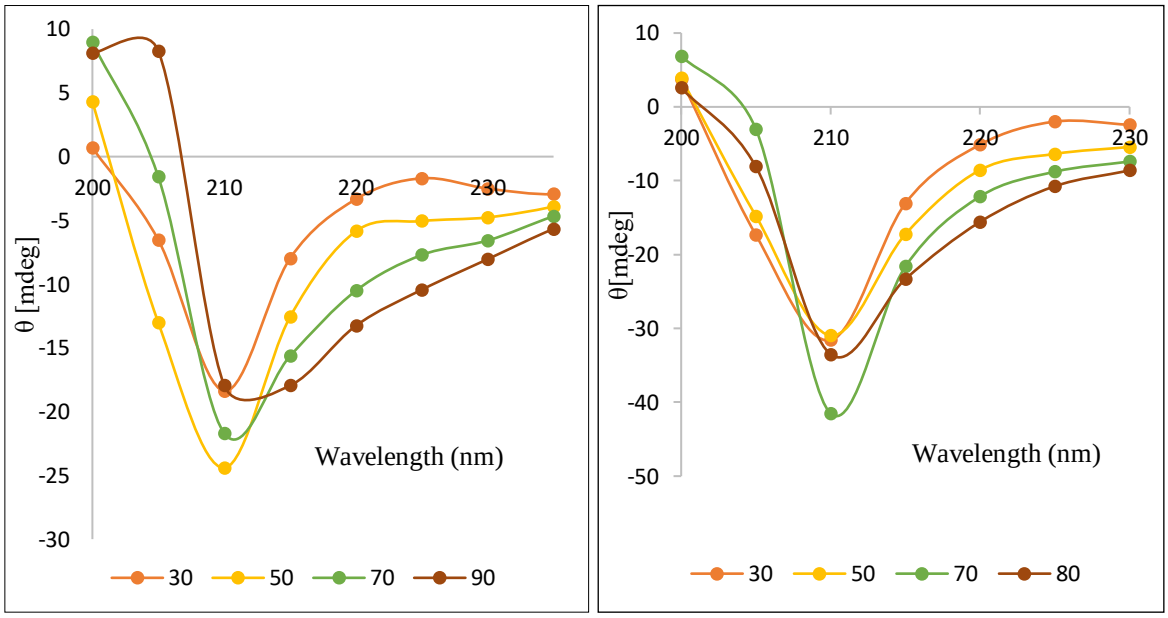
These results suggested that enzyme undergoes changes in the secondary structure, particularly in α-helix. Extremely high temperature leads to irreversible inactivation of the enzyme due to deformation in the protein structure and loss of catalytic site. Batumalaie et al. (2018) also reported that the secondary structure of lipase from *Acinetobacter haemolyticus* is denatured due to high temperature treatment.

4.3.5. Thermal reversibility/irreversibility of lipase by CD spectra

The reversibility/ irreversibility of thermal denaturation was further verified by giving temperature treatment for 30 min under different temperature 50 and 70°C for *P. punonensis* and 30, 60, 80°C for *B. gibsonii* as shown in Fig 4.9 (a,b) and 4.10 (a,b,c) respectively. Thereafter the enzyme samples were placed at room temperature (30°C) to see the time dependent changes in the secondary structure of the enzyme. The CD spectra of untreated control of both strains lipase (30°C) were recorded and compared with the CD spectra of treated samples of *P. punonensis* (50°C and 70°C) and *B. gibsonii* (60 and 80°C) lipase kept at room temperature for recording the time dependent recovery in the secondary structure of protein. The results of CD spectra of *P. punonensis* control and treated samples (Fig 4.9 a, b, c) revealed that a negative peak α-helix observed at 210 nm gradual negative increase in the intensity in a time dependent manner in the sample treated at 50°C. However, treatment of sample at 70°C resulted into initial increase in the intensity after 30 min and thereafter it remained unaffected with increasing time of cooling at room temperature. These results suggested deformation in the α-helix of protein at 50°C with no sign of reversibility of conformational structure of enzyme. However, α-helix of the enzyme treated at 70°C showed initial deformation in the protein after 30 min of cooling, but no subsequent changes in peak intensity at 210 nm with respect to time of cooling suggested for high temperature induced stable deformation in the secondary structure of protein. The

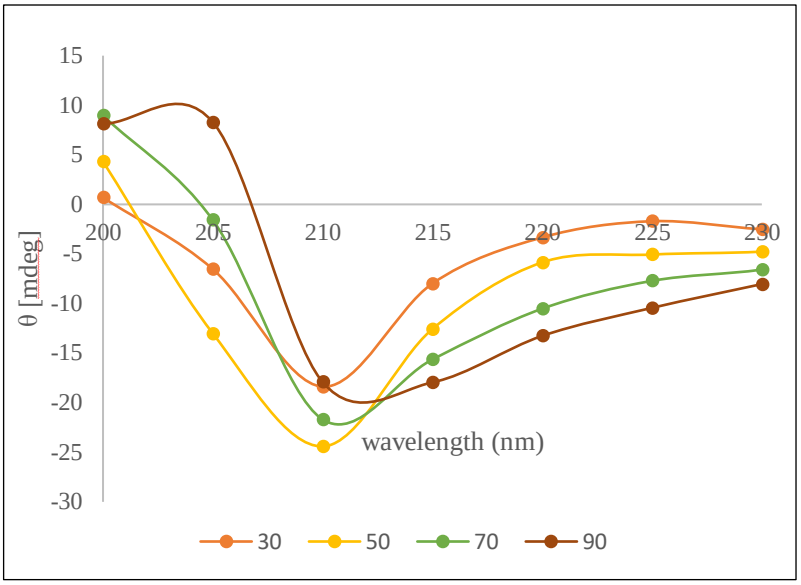
overall results indicated irreversible inactivation and deformation in the secondary structure of enzyme due to temperature treatment.

The results of CD spectra of *B. gibsonii* control and treated samples (Fig 4.10 a, b, c, d) revealed negative peaks of α-helix observed gradual negative increase in the intensity in a time dependent manner in the sample treated at 60°C. However, treatment of sample at 30°C resulted into constant after temperature treatment. The above result suggested that deformation in α-helix of protein at 60°C was reversible to its original position as shown in untreated, which suggested the reversible conformational structure of enzyme at 60°C.

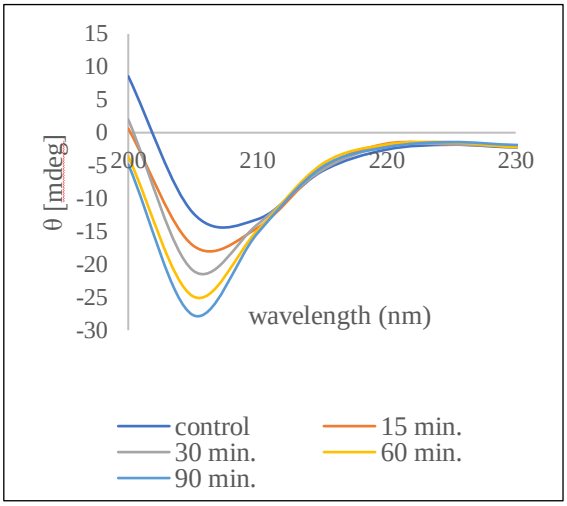


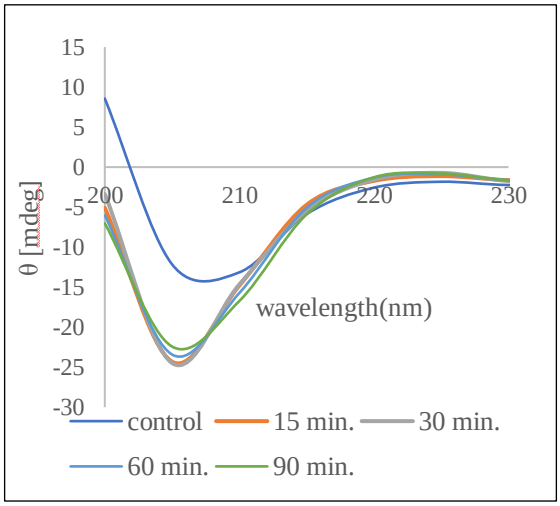
(a) *P. punonensis* **(b) *B. gibsonii***

Fig 4.8 (a,b) Far UV CD spectra of (a) *P. punonensis* and (b) *B. gibsonii* lipase showing the CD spectra of protein for increasing temperature



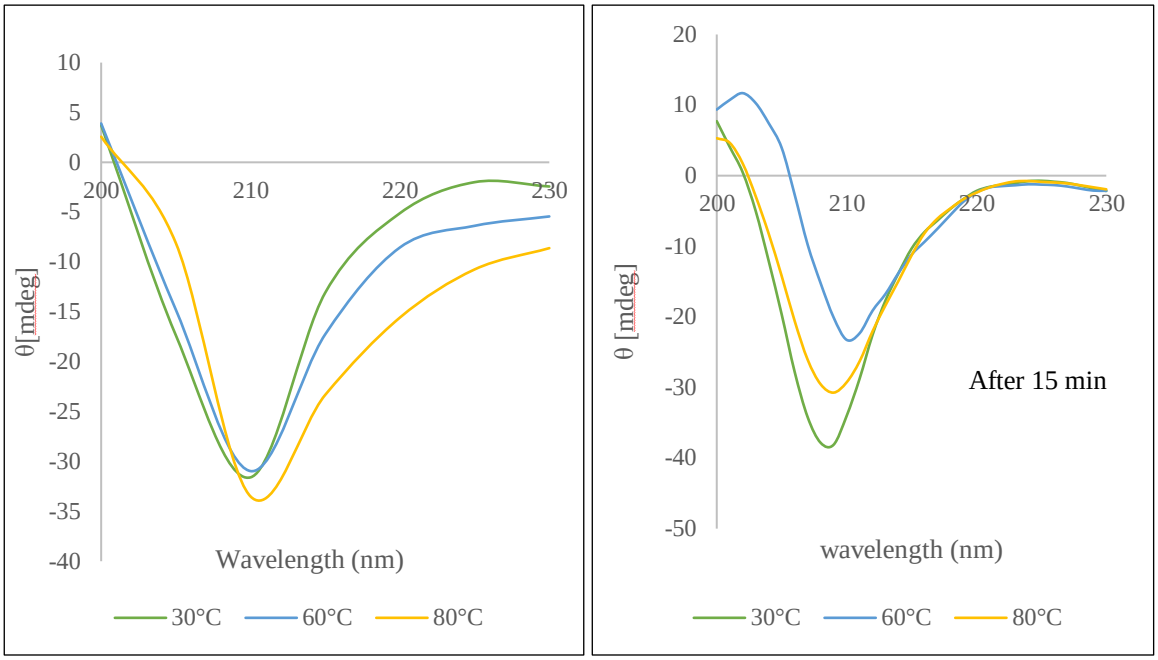
(a)



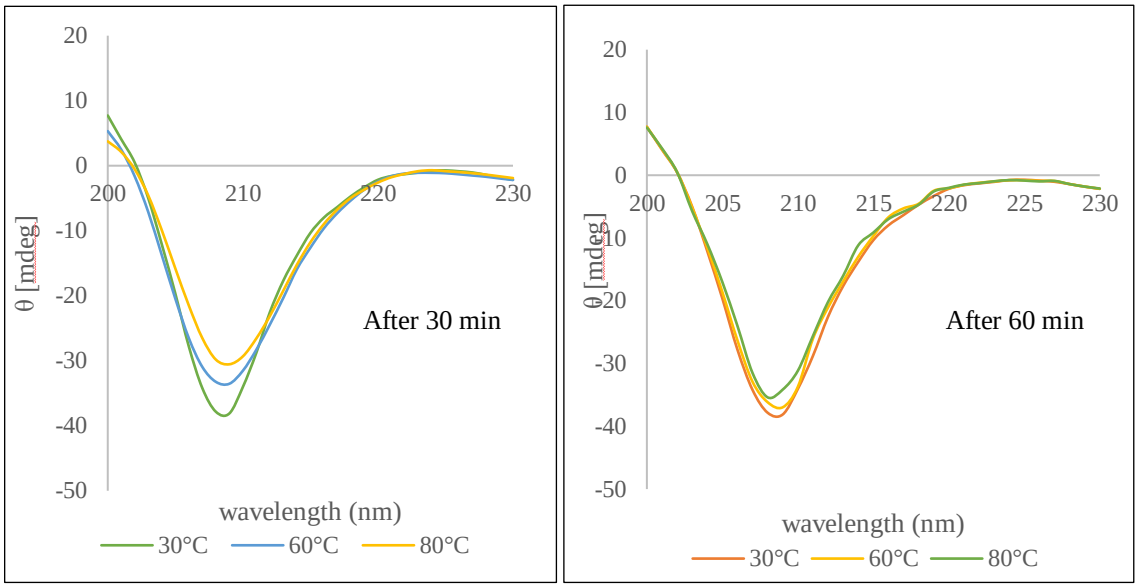


(b) **(c)**

Fig 4.9 (a, b, c) Far UV-CD spectra of the refolded protein recorded at room temperature after heating it for 30 min. (a) untreated (b) 50 ˚C and (c) 70 ˚C at different time intervals for *P. punonensis*



(a) **(b)**



(c) **(d)**

Fig 4.10 Far UV-CD spectra of the refolded protein recorded at room temperature after heating it for 30 min. (a) untreated (b) after 15 min of incubation (c) after 30 min of incubation and (d) after 60 min of incubation at different temperatures for *B. gibsonii* lipase

4.4 Conclusion

The present investigation relates to the kinetic and thermodynamic evaluation of alkaline lipase from psychrotolerant *P. punonensis* and halotolerant *B. gibsonii*. The study revealed maximum lipase activity for *P. punonensis* and *B. gibsonii* was found at 30°C and pH 9.0 and 60 °C and pH 9.0 respectively. It was observed that *P. punonensis* enzyme activity was stimulated in the presence of surfactant SDS, protease inhibitor PMSF, metal chelator EDTA whereas, *B. gibsonii* lipase activity was enhance by SDS, protein inhibitor PMSF, EDTA and Al^{+3}. Kinetic parameters (K_m, V_{max} and k_{cat}) of the enzyme exhibited maximum catalytic efficiency at 30°C for psychrotolerant and 60°C for halotolerant. A rise in temperature from 50-70°C in *P. punonensis* lipase led to gradual thermal inactivation as indicated by kd value and half-life of enzyme inactivation. Whereas, *B. gibsonii* lipase activity was spontaneous and kinetically favourable up to 60°C. Thermodynamic constant value of *P. punonensis* lipase revealed that enzyme catalysis was spontaneous and efficient at 30°C as compared with 50 and 70°C. Whereas, it was thermostable up to 60°C for *B. gibsonii*. These findings suggest that this alkaline lipase could be beneficial for detergent and commercial purpose by various industries.

CHAPTER 5

CHAPTER 5

BIODEGRADABILITY AND BIODEGRADATION PATHWAYS OF DI(2-ETHYLHEXYL) PHTHALATE BY TWO EXTREMOPHILIC BACTERIAL STRAINS

5.1. Introduction

Phthalic acid esters (PAEs), as alkyl aryl or di-alkyl esters of 1,2-benzenedicarboxylic acid, are the artificial compounds which are widely used as plasticizers in the industrial, medical and many other household products to improve the flexibility of polymeric materials. These are commonly used due to their excellent properties to improve the flexibility, durability and longevity of plastics (Xu et al., 2008). PAEs are used as additive in polyvinyl chloride as products, paints, pesticides and medical and food packaging material products by 50% of its mass (Xu et al., 2014). It has been estimated that annual production of PAEs surpasses 6 million tons per year (Abdel Daiem et al., 2012). However, large amount of phthalate is also widely discharged into water bodies by many industries, which raise the concern about human and animal health (Li et al., 2019). PAEs can easily contaminate the water bodies due to their strong bond formation with the plastic products. They are also migrating into air due to their relatively high vapour pressure (Xu et al., 2017). Several PAEs have been reported as endocrine disruptors in human as well as reproductive toxicants in animals (Wang et al., 2012; Wang and Fan, 2014).

More than 60 types of PAEs are produced and consumed, but DEHP is one of the most widely used plasticizer in plastic products due to their strength, flexibility, stability and temperature tolerance (El-Shinnawy, 2016). It can easily migrate into environment due to their non-chemical bond formation and high octanol/water partition coefficient (Benjamin et al., 2015 and Zhao et al., 2017). These are also considered as potential human

carcinogens and important environmental pollutants by China National Environmental Monitoring Centre (Lü et al., 2018) and US Environmental Protection Agency (USEPA, 2007). Several reports showed the presence of phthalates in human blood, urine and breast milk of people who have been exposed to hazards of plastic industry (Hogberg et al., 2007). The leaching capability of DEHP is faster in the environment due to absence of chemical bonding between polymer mesh and the plasticizer (Pradeep et al., 2015). It can be easily accumulated in plants including vegetables and crops like rice, which is again a potential health hazard to humans (Sun et al., 2015, Cai et al., 2015). DEHP is considered as an important pollutant by international environmental regulatory agencies due to high perseverance in the environment and biological toxicity to living bodies (Xu et al., 2014).

The biodegradation of different types of phthalates in wastewater, soil, sludge, and river sediment has been extensively investigated (Abdel Daiem et al., 2012), however, most of the research were related to dialkyl phthalates degradation with short side-chain. The long side-chain phthalate esters were studied less due to their difficulty in biodegradation (Wang et al., 2015; He et al., 2018). DEHP can be removed from environment by the metabolic pathways of bacteria, fungi, algae and even yeasts cells under aerobic and anaerobic conditions (Gao et al., 2016 and Li et al., 2020). Hence, biodegradation of DEHP is one of the major concerns of environmentalists.

Several DEHP degrading bacterial strains were isolated and characterized such as, *Agromyces* sp. MT-O (Ahuactzin-Pérez et al., 2016), *Fusarium culmorum* (Zhao et al., 2016a, 2017), *Pseudomonas* (Singh et al., 2017), *Rhodococcus ruber YC-YT1* (Yang et al., 2018), *Gordonia* sp. strain 5F (Huang et al., 2019), *B. mojavensis* B1811 (Zhang et al., 2018), *Rhodococcus pyridinivorans* XB (Zhao et al., 2018), *Acinetobacter* sp. strain LMB-5 (Yue et al., 2017), *Agromyces* sp. MTO strain (Zhao et al., 2016), *Rhizobium* sp. LMB-1 (Tang et al., 2016), *Microbacterium* sp. J-1(Zhao et al., 2017), and *Acinetobacter* sp. SN13

(Xu et al., 2017). These bacterial strains were isolated from different sources including vegetable soil from greenhouse, landfill soil, and plastic waste debris and activated sludge. Until now, little literature is available on DEHP biodegradation with effects of carbon/ nitrogen source.

Several studies on different degradation pathways of PAEs to phthalate monoesters involve de-esterification or de-alkylation, β-oxidation and trans-esterification, which lead to primary degradation of PAEs to phthalate monoester, which may be further degraded into phthalic acid (Liang et al., 2008). Generally, DEHP has longer side chain, hence, its degradation takes longer time than other short chain PAEs compound such as, di-n-butyl phthalate, dimethyl phthalate and diethyl phthalate (Chang et al., 2004; Meng et al., 2014; Wen et al., 2016). The biodegradation pathways start with the conversion of DEHP into mono (2-ethylhexyl) phthalate (MEHP), after that MEHP is further hydrolysed to phthalic acid (PA) and then CO_2 and/or CH_4 (Benjamin et al., 2016, Staples et al., 1997). The phthalic acid degradation often follows two dioxygenase catalysed pathways under aerobic conditions and produce a common intermediate product protocatechuate, which is also known as 3,4-dihydroxy benzoate. It is known that Protocatechuate is generally processed via ortho or meta cleavage pathway. Under the anaerobic degradation pathway, the phthalic acid is degraded to benzoate, which is subsequently cleaved into hydrogen, carbon dioxide and acetate via β-oxidation, (Liang et al., 2008). The intermediate products formed during degradation were detected by HPLC or GC-MS (Shailaja et al., 2008; Zhao et al., 2016a, 2017).

In the present study, two lipases producing extremophilic psychrotolerant and halotolerant bacteria were isolated form soil and water samples, respectively. Both psychrotolerant *P. punonensis* and halotolerant *B. gibsonii* were capable of degrading the PAEs, especially the DEHP. The DEHP degradation efficiency and optimal conditions for biodegradation of

DEHP by these bacterial strains were investigated to establish their potential application in environmental clean-up of phthalate pollutants. Further, based on the intermediates of DEHP biodegradation pathways in these bacterial strains, we have proposed two different biodegradation pathways for DEHP by *P. punonensis* and *B. gibsonii* under aerobic conditions.

5.2. Material and methods

5.2.1. Culture media for bacterial growth

Psychrotolerant *P. punonensis* was isolated from soil sample of Leh (Laddakh), J&K, India, while *B. gibsonii* was isolated from water samples collected from coastal area of Dwaraka, Gujarat, (India). The isolated bacterial strains were grown on modified NA. The culture medium consists of (g/L): Beef extract 1.5, Yeast extract 1.5, Peptone 5.0, NaCl 5.0, Agar 15.0 at pH 7.2±0.2 for psychrotolernat, while NaCl 14.0 was used for halo-tolerant strain. The pH of the medium was adjusted by using HCl or NaOH solutions before autoclaving these at 121°C for 20 min.

5.2.2. Substrate utilization test

Both the isolated bacterial strains, cultured in modified nutrient broth medium for 24 hr, were used as inoculum (0.2%) to inoculate 100 mL bacterial culture (2%). The DEHP utilization was checked by inoculating bacterial strain in the culture broth containing 500 mg/L of DEHP as sole source of carbon substrate. The flasks were incubated at 30°C on a rotatory shaker at 180 rpm. DEHP utilization by bacteria was monitored by measuring the growth of bacteria and quantification of residual DEHP in a 5 days old culture. The biomass growth of bacteria was assessed by measuring the optical density at 600 nm by a double beam UV-Visible spectrophotometer after 36 hr of incubation. A sterile control was also used without DEHP. All the DEHP utilization experiments were conducted in triplicates.

5.2.3. Quantification of residual DEHP concentration

The residual DEHP concentration was estimated by adding 20 mL dichloromethane into supernatant of culture media. The dichloromethane containing organic phase, which was extracted from aqueous phase thrice (20 mL, 3X), then the solvent dichloromethane was dried with nitrogen gas. The remaining residue was dissolved in 5mL of methanol was filtered through using 0.22 μm sterile membrane. The sample were stored at -20°C for high-performance liquid chromatography (HPLC) analysis (Zhang et al., 2020).

5.2.4. DEHP biodegradation kinetics and influencing factors

To investigate the influence of the initial concentration of DEHP on the biodegradation, different initial DEHP concentrations (100, 250, 500, 750, and 1000 mg/L) were investigated for the degradation by *P. punonensis* and *B. gibsonii*. DEHP biodegradation by both the bacterial strains is assumed to fit to the following first-order kinetic equation:

$$\ln C = -Kt + A \quad (5.1)$$

Where C = DEHP concentration in mg/L,

t = time,

K = first order rate constant

A = constant

The half-life of DEHP biodegradation by both bacterial strains are calculated by following equation:

$$t_{1/2} = \ln 2/K \quad (5.2)$$

Some physico-chemical factors such as pH and temperature play a major role in the biodegradation of DEHP. The role of pH and temperature were investigated to determine the optimal conditions for DEHP biodegradation. A range of pH (pH 5.0 to 10.0) and temperature (10-50°C) conditions were selected to determine their effects on DEHP

degradation efficiency. The biodegradation of DEHP by *P. punonensis* and *B. gibsonii* were conducted under different concentrations of DEHP (100, 250, 500, 750, 1000 mg/L). The DEHP degradation kinetics parameters associated with degradation in both the strains were estimated by Gompertz model (Li et al., 2019) as given below:

$$S = S_0 \left\{ 1 - \exp\left[-\exp\left(\frac{eRm}{S_0}(t_0 - t) + 1 \right) \right] \right\} \quad (5.3)$$

Where, S= substrate concentration in mg/L,

S_0 = initial substrate concentration in mg/L,

R_m = Maximum substrate transformation rate in mg/L/h,

t_0 = lag phase time (hr)

t = time of incubation (hr)

5.2.5. HPLC analysis methods

The DEHP residual concentration in the samples were calculated by using an HPLC (Shimadzu class VP.V6.10) equipped with a Chiralpak IB column (4.6×250mm, 5.0µm). A well-separated compounds profile was obtained by gradient elution. Mobile phases were used as formic acid (A), ultrapure water (B) and acetonitrile (C). The sample volume 20 µL was taken as injection volume and ratio of mobile phase was maintained as 9:1 by comprising of acetonitrile and deionized water. The flow rate of eluent was 0.5 mL/min. the linearity of the calibration curve was assessed over the range from 1 to 100 mg/L (DEHP), in triplicates by the external standard method with a correlation coefficient equal to 0.995. The column temperature was maintained at 25°C throughout the run. Qualitative data were obtained by comparing the sample peaks with peaks of the standard compounds, and the concentration of DEHP in the samples was calculated using external standards (Montero et al., 2017).

5.2.6. Identification of metabolites during DEHP degradation

In order to analyze the metabolites of DEHP biodegradation, the aliquots were withdrawn every 8 hr during the 5 days incubation and the samples were acidified to pH 2.0, and then DEHP was extracted thrice by using ethyl acetate. The ethyl acetate extract from the cell free culture medium was filtered through a sterile membrane (0.22 μ) for analysis by GC/MS. The peaks assigned to degradation intermediate compounds were detected on HPLC chromatogram and the same were confirmed by combination of techniques including Gas chromatography-mass spectrometry (GC/MS/MS), Triple Quadrupole Mass analyzer (Model Name GC1310 /TSQ8000 Evo system) with liquid auto sampler- Triplus RSH, by Thermofischer Scientific Pvt Ltd. Mumbai (India). The column condition was taken as 40°C/5 min, Ramp rate 10°C/min - 260°C/10 min, Injector -260°C, Ion source- 200°C, Interface-260°C. The mass range were taken as 0-400 and injection volume was taken as 1.0 μL. The mass spectrum of the unknown compounds was compared with the spectrum of the known compounds by using the database of National Institute Standard and Technology (NIST).

Statistical analysis

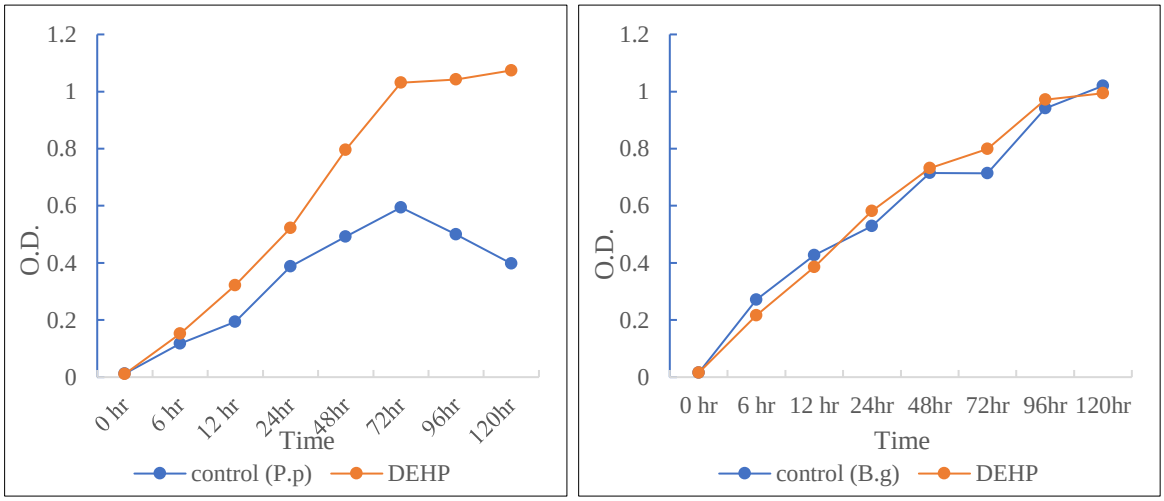
The statistical analysis was done by using one-way analysis of variance (ANOVA) (SPSS 20).

5.3. Results and discussion

5.3.1. Growth and DEHP utilization by extremophilic bacteria

In the present study biodegradation of DEHP was studied by both isolated bacterial strains *P. punonensis* and *B. gibsonii*. The association between bacteria and DEHP was more than expected. The biomass production was found to be higher in case of *P. punonensis*, whereas

it was found to be similar to control in *B. gibsonii* when DEHP was used as a sole carbon source in the culture media. Chang et al. (2007) reported that some co-substrates like yeast extract, humic acid and cellulose could enhance the cellular growth when added in culture medium. Both, yeast extract or beef extract were also used as a co-substrate in the medium to enhance the biodegradability of DEHP. The DEHP substrate utilization test was carried out in nutrient broth containing 500 mg/L of DEHP, where a control set was run by using 2 % bacterial inoculum without DEHP. At the time of DEHP addition in the culture medium, it was ensured that the bacterial cell density was sufficient for DEHP biodegradation. Earlier workers have shown that DEHP acts as growth stimulant for bacterial growth (Li et al., 2019). As depicted in Fig. 5.1a, growth of *P. punonensis* was stimulated by about 170% with addition of DEHP as an additional carbon source, when compared with the bacterial growth without DEHP (100%). On the other hand, growth of *B. gibsonii* (Fig. 5.1b) remained almost unaffected due to addition of DEHP as additional carbon source. These results clearly suggest that DEHP dependent increase in the growth of *P. punonensis* was perhaps due to its effective ability to utilize DEHP as carbon source, whereas absence of any positive increase in the growth of *B. gibsonii* in the presence DEHP indicated an incomplete assimilation of DEHP. Further, we cannot rule out the possibility that both the bacterial strains use different metabolic pathways to metabolize the DEHP.



(a) **(b)**

Fig.5. 1. Growth of bacteria in presence of substrate DEHP (500 mg/L) for utilization test by both bacterial strains. (a) *P. punonensis* (b) *B. gibsonii*

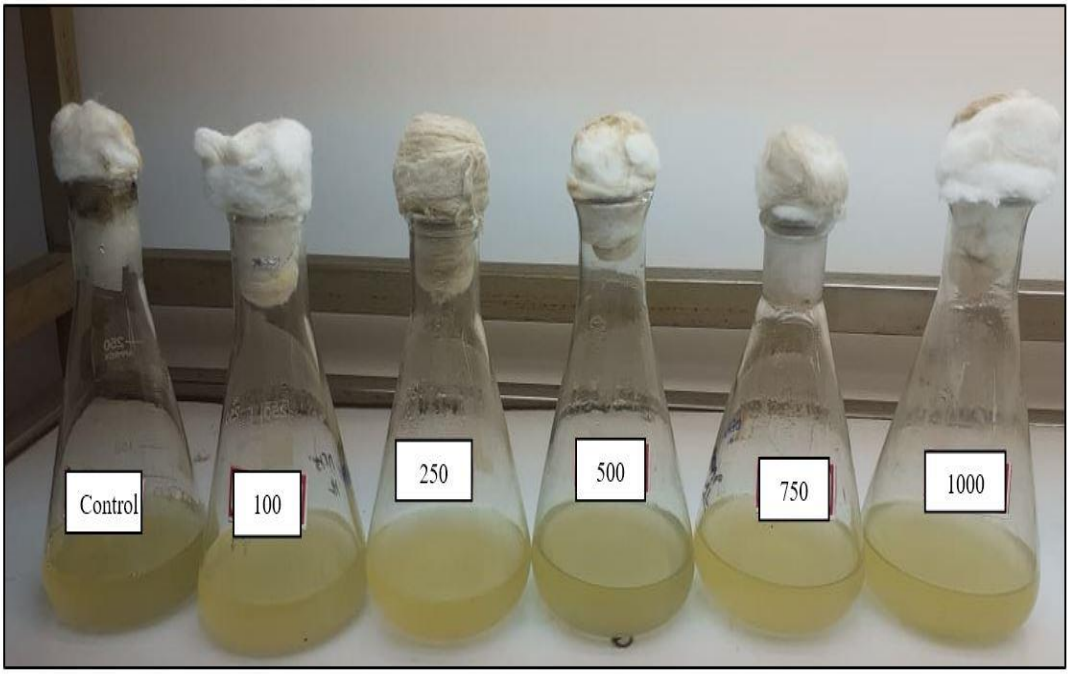
5.3.2. Effects of pH and temperature on DEHP degradation

The DEHP degradation by *P. punonensis* and *B. gibsonii* was studied in response to varying pH conditions (pH 5.0-10.0) as shown in fig. 5.3a. The results showed pH dependent initial increase in the DEHP degradation by both *P. punonensis* and *B. gibsonii* up to pH 7.0. *B. gibsonii* exhibited higher rate of DEHP degradation than *P. punonensis* in the acidic range. In the alkaline range between pH 7.0 to 10.0, the biodegradation of DEHP by both the strains declined by approximately 91- 95% between pH (5.0-9.0). These results also revealed that *P. punonensis* had narrow range of optimum pH for DEHP degradation, whereas *B. gibsonii* showed a broad range of pH conditions (5.0-9.0) for DEHP degradation. Further, ratio of DEHP degradation by *B. gibsonii* under all the pH conditions (pH 5.0-9.0) did not vary with respect to initial pH value, indicating that the pH might not be an important factor for degradation of DEHP by *B. gibsonii*. Earlier report suggested that accumulation of intermediate metabolites reduce the pH of culture medium (Nahurira et al., 2017). In the present study, it was observed that pH value of the culture medium

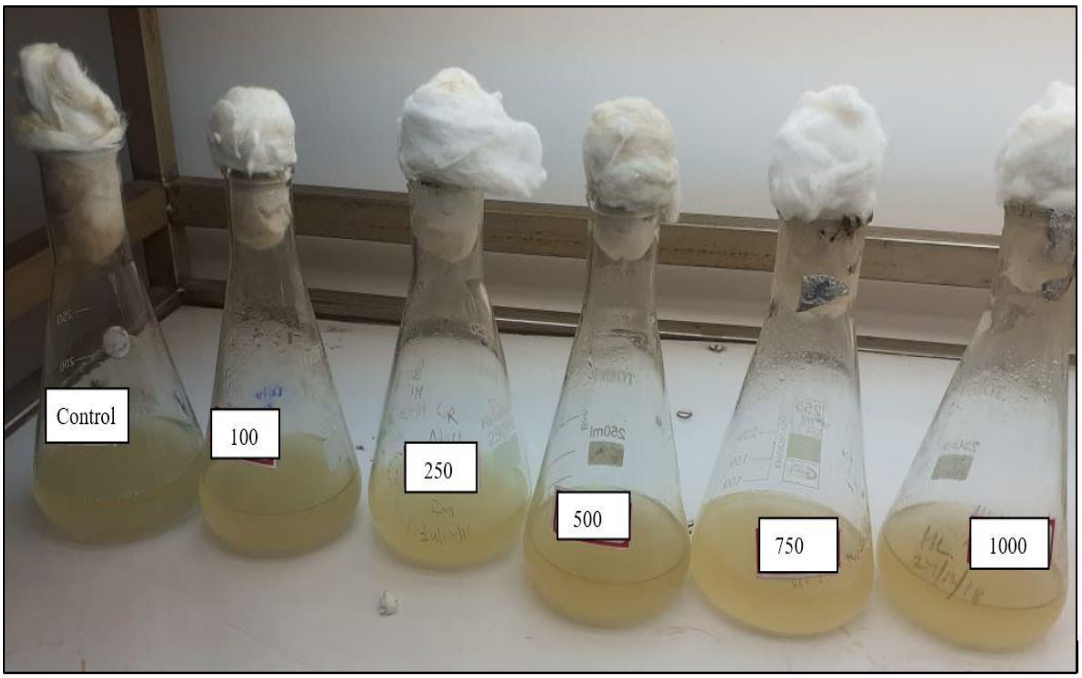
remained unchanged even after accumulation of intermediate metabolites of DEHP degradation. In view of these results, pH 7.0 was selected as an optimal pH for further study on DEHP degradation by both the bacterial strains.

Since the temperature plays an important role in the enzymatic degradation of phthalate degradation, a range of temperature conditions (10-50°C) were selected to examine the effect of temperature on DEHP degradation. The results (fig.5.3 b) revealed that initially the rate of DEHP degradation by *P. punonensis* increased from 47 to 92 % with rise in temperature from 10 to 30°C. As the temperature was raised to 50°C, the degradation rate of DEHP declined to 24 %. These results showed that the optimum rate of DEHP degradation was obtained at 30°C.

The results on effect of temperature on DEHP degradation in *B. gibsonii* revealed that lower temperatures (10-20°C) did not exhibit very good rate of DEHP degradation (24-41%) as compared to *P. punonensis*, whereas the rate of DEHP degradation at room temperature (30°C) was increased by about 96 %. A further increase in the temperature from 30-50 °C led to decline in the rate of DEHP degradation from 96 to 74 %. The overall results exhibited that the optimal temperature for phthalate degradation by both the bacterial strains was 30 °C.



(a)



(b)

Fig. 5.2. Different concentration of DEHP used in mg/L for degradation by (a) *P. punonensis* and (b) *B. gibsonii*

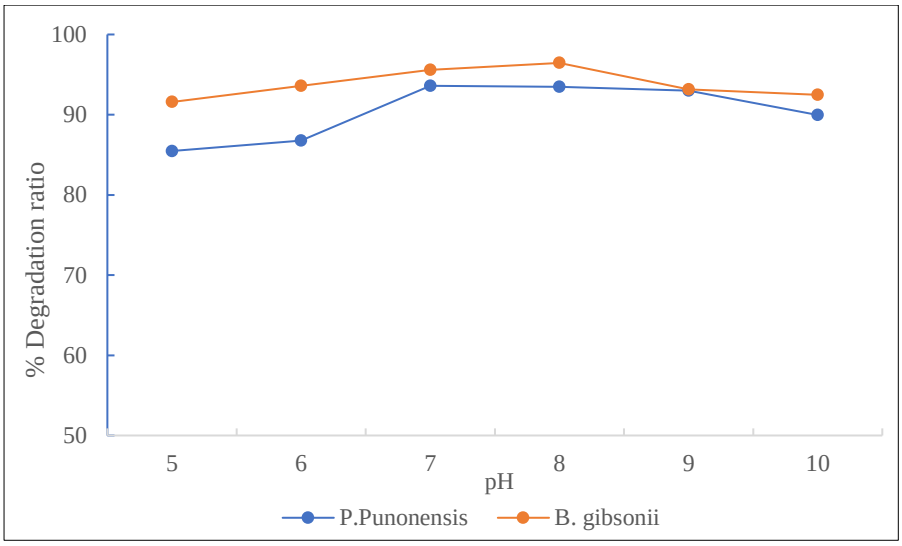


Fig.5.3 a. Effects of initial pH on degradation of DEHP (500 mg/L) by *P. punonensis* and *B. gibsonii*. The measurements were taken after 120 hr of incubation.

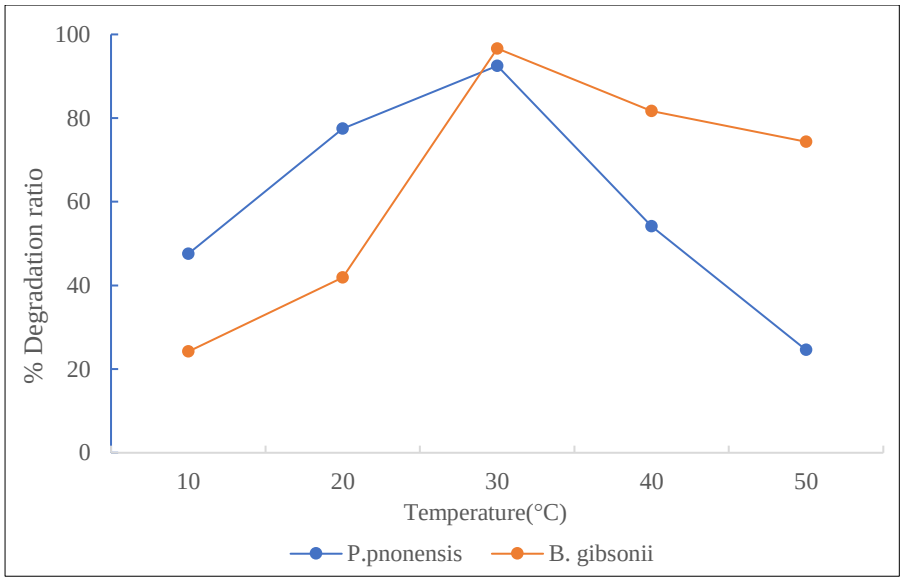
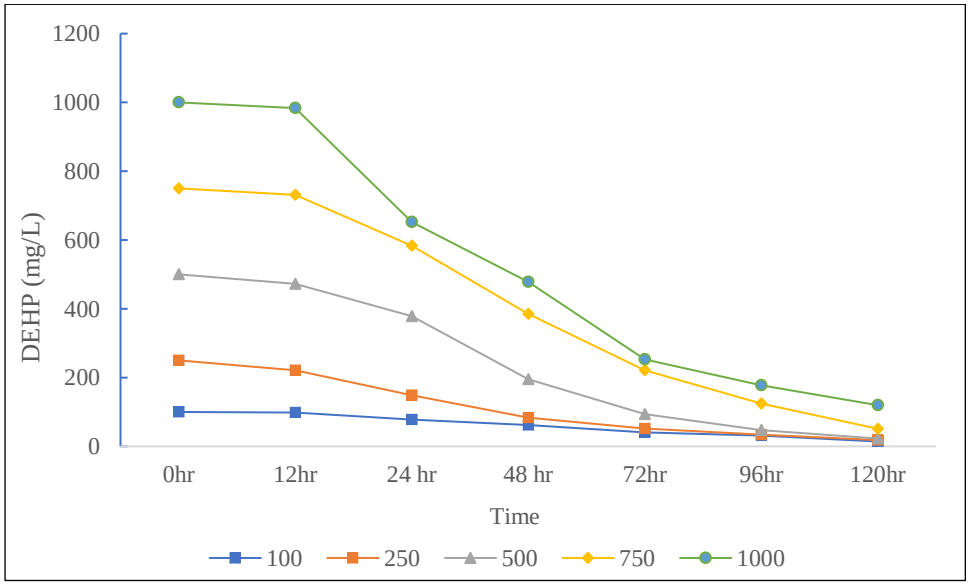


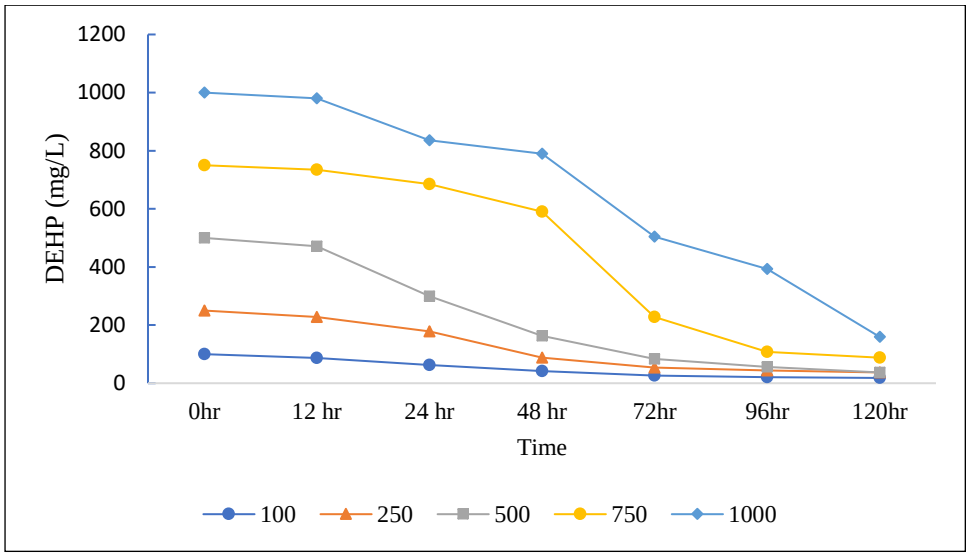
Fig 5.3. b. Effects of temperature on degradation of DEHP (500 mg/L) by *P. punonensis* and *B. gibsonii*. The measurements were taken after 120 hr of incubation.

5.3.3. Effects of DEHP concentrations on its biodegradation

The initial concentrations of DEHP (100 mg/L, 250 mg/L, 500 mg/L, 750 mg/L and 1000 mg/L) were taken to study the effect of substrate concentrations on biodegradation of DEHP. The results showed an initial sluggish phase of around 12 hr in DEHP biodegradation, followed by a rapid phase of biodegradation in case of both the strains (between 12 to 120 hr). The results depicted the presence of very small residual DEHP after 120 hr of incubation. The concentration dependent biodegradation of DEHP by *B. gibsonii* and *P. punonensis* are shown in the Fig 5.4 (a) and (b) respectively. The results revealed that the degradation of DEHP was enhanced with time and was found to be maximum at 500 mg/L after 120 hr which were 92.59 % and 95.44 % for *B. gibsonii* and *P. punonensis*, respectively. Whereas, the degradation rate was found to be 84.05 and 88.03% for *B. gibsonii* and *P. punonensis* at 1000 ppm after 120 hr of incubation.



(a)



(b)

Fig 5.4. Effect of initial concentration of DEHP on the biodegradation by (a) *P. punonensis* and (b) *B. gibsonii*

The degradation kinetics of both isolated bacterial strains were studied by Gomertz model (Jin et al., 2012). Generally, Gompertz model was used to describe sigmoidal curves during degradation. An apparent lag phase was detected during DEHP degradation by Gompertz model and it was found that substrate can be rapidly degraded by both strains. According to Gompertz model, the competitive inhibition between the compounds and the number of dominant bacteria were seen. In this study, no apparent lag phase for DEHP degradation indicated that both strains were able to adapt quickly to the DEHP contaminated environment.

The DEHP biodegradation kinetics were studied at different initial DEHP concentration was analysed by Gompertz model. The initial rate of DEHP degradation by *B. gibsonii* between 10 to 36 h increased from 4.79 mg/L/h to 37.31 mg/L/h. In case of *P. punonensis* the biodegradation rate between 7 to 22 hr increased from 7.85 mg/L/h to 24.59 mg/L/h. It has been reported by previous researchers that *Arthrobacter* sp. can degrade 52% of DEHP after 72 hr of incubation (Wen et al., 2016). The DEHP degradation in *Sphigomonas* sp.

and *Corynebacterium* sp. strains after 168 hr of incubation (Chang et al., 2004) was about 11.5% and 11.2%, respectively. *Burkholderia pyrrocinia* B1213 sp. could degrade about 98% of DEHP after 144 hr of incubation (Li et al., 2019). Meng et al. (2014) reported that *Pseudoxanthomonas* sp. could also degrade about 30 % of DEHP (1250mg/L). It was observed that *Bacillus mojavensis* B1811 could completely degrade DEHP after 96 hr incubation (Zhang et al., 2018). *Rhodococcus pyridinivorans* XB removed 98% of DEHP after 48 hr of incubation (Zhao et al., 2018). The kinetics parameters t_0 (lag phase) of DEHP at 100 mg/L was determined to be 7.56 and 10.4 for *Pseudomonas* sp. and *Bacillus* sp. respectively (Table 5.2). Sarkar et al. (2013) reported that many bacteria produced bio-surfactants play an important role in the degradation of numerous hydrophobic compounds. Bio-surfactants have ability to increase the solubility of DEHP in culture media and enhance the binding area between the bacteria and DEHP. Bio-surfactants could have the capability to increase the degradation rate of hydrophobic compounds. In the present study, it was observed that both *Pseudomonas* and *Bacillus* strains exhibited a remarkable capability to degrade long chain phthalate (DEHP), over a wide range of conditions, and absence of intermediate accumulation of intermediated during the biodegradation makes both the strains as ideal microbes for bioremediation of DEHP.

Table 5.1. Kinetic parameters of DEHP degradation by extremophilic bacterial strains with different initial concentrations

DEHP concentration (mg/L)	t_0 (hr) *B. gibsonii*	t_0 (hr) *P. punonensis*	R_m (mg/L/h) *B. gibsonii*	R_m (mg/L/h) *P. punonensis*
100	10.4	7.56	4.79	7.85
250	11.8	14.76	7.03	11.05
500	18.63	22.06	16.35	19.86
750	32.56	20.97	23.92	21.66
1000	36.40	18.54	37.31	24.59

5.3.4. Identification of DEHP metabolites

To explore the degradation pathways of DEHP degradation by *P. punonensis* and *B. gibsonii*, the metabolites of longer chain phthalate (DEHP) were identified by GC-MS. A standard graph of DEHP was prepared using 0 to 100 mg/L concentration of standard compound as shown in Figure 5.6. The below mentioned graph depicts standard calibration plot of DEHP.

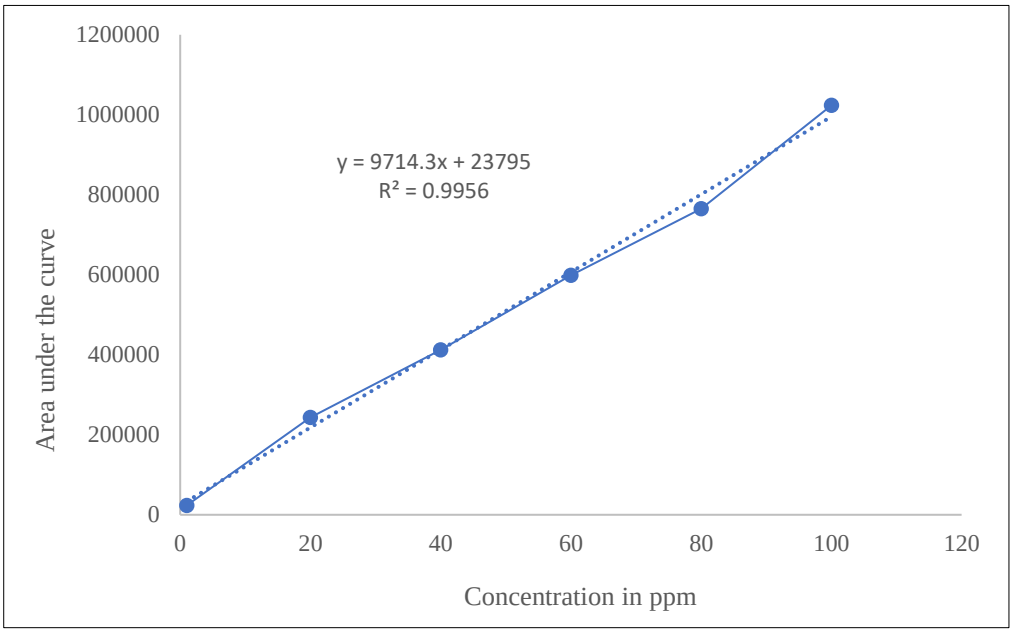


Fig. 5.5. DEHP Standard calibration graph

The following straight-line equation was obtained from Calibration plot:

$$Y=9714.3x+2.3795$$

Keeping the area under the curve relevant to retention time 3.1-3.7 min in the place of Y the value of x (residual concentration of DEHP) was calculated as given below (Table 5.3):

Table 5.2. Residual concentration of DEHP after 3rd and 5th day of incubation

Concentration	*B. gibsonii*		*P. punonensis*	
	3rd day	5th day	3rd day	5th day
100 ppm	56.45 ppm	48.20 ppm	90.66 ppm	31.06 ppm
300 ppm	53.36 ppm	37.02 ppm	30.54 ppm	2.26 ppm
500 ppm	55.93 ppm	37.02 ppm	22.76 ppm	22.76 ppm
800 ppm	58.363 ppm	37.96 ppm	28.80 ppm	21.2 ppm
1000 ppm	303.90 ppm	51.45 ppm	35.44 ppm	19.67 ppm

The residual concentration of DEHP was used for GC-MS analysis of intermediated metabolites formed during the degradation process. It was found that the first metabolite was identified as MEHP (m/z 277) during the biodegradation of DEHP by both the bacterial strains (Table 5.4). MEHP polarity was high and its molecular weight was less than DEHP thus its retention time was less than DEHP. The above degradation pathway proposed by many researchers confirmed that DEHP was first degraded to MEHP which was then hydrolysed to PA (Ahn et al., 2006, Benjamin et al., 2016, Nahurira et al., 2017, Xu et al., 2017 and Zhao et al., 2016). However, one peak of MBP (monobutyl phthalate) (m/z 221) was identified, which indicated involvement of some alternative pathway for DEHP degradation by *B. gibsonii*. Some previous studies showed that esterase was responsible for degradation of DEHP to MEHP (Li et al., 2019). The results, thus, suggested that the alkyl chain of MEHP was transformed into MBP and then to PA by *B. gibsonii* and *P. punonensis.* This degradation pathway was apparently different from the previously reported degradation pathways, where MEHP is directly converted into PA by MEHP hydrolase (Li et al., 2019). The alkyl chain in MEHP was broken by these bacterial strains to produce MBP through β-oxidation. The previous studies also suggested that the biodegradation pathways of DEHP includes the conversion of PA degraded into PCA by

aerobic degradation (Singh et al., 2017). The PCA was further degraded into oxaloacetate and pyruvate. However, anaerobic degradation led the degradation of pyruvate into benzoate, CO_2 and acetate. These two extremophilic bacterial strains are aerobic bacteria, and PCA, oxaloacetate and pyruvate were not detected during the biodegradation of DEHP. It was observed that a new substance with chemical formula $C_6H_{10}O_3$ was detected (m/z 129.1371) which may be 4-oxo-hexanoic acid or 3-oxo-hexanoic acid or 5-oxo-hexanoic acid. The chemical structure of 4-oxo-hexanoic acid was found to be closer to β-ketoadipic acid which is also known as β-ketoadipate or 3-ketoadipate. The β-ketoadipic acid is one of the main intermediate products during the microbial degradation of aromatic compounds (Yamanashi et al., 2015, Harwood and Parales, 1996; Okamura-Abe et al., 2016). Xu et al. (2017) suggested that β-katoadipate is formed from PCA via β-ketoadipic enollactone and dioxygenase enzyme when *Acinetobacter* sp. SN13 was used for biodegradation. Hence, it was inferred that 4-oxo-hexanoic acid was formed by the β-ketoadipic acid during DEHP degradation process of biological metabolism. Therefore, 4-oxo-hexanoic acid formation in both extremophilic bacterial strains has possibly taken place during the conversion of PA to PCA. PCA as an aromatic compound, which was quickly converted to β-ketoadipic acid, and then to 4-oxo-hexanoic acid.

Table 5.3 GC-MS full scan acquisition for the identification of DEHP biodegradation intermediates

Acronym Compound	Elemental formula	Experimental mass (m/z)	Calculated mass (m/z)	Error (ppm)	RT (min.)
MEHP	$C_{16}H_{22}O_4$	277.1449	277.1443	-0.73	1.01
MBP	$C_{12}H_{14}O_4$	221.0829	221.0822	-0.62	0.87
PA	$C_8H_6O_4$	165.0118	165.0109	-0.41	0.56
4-oxo-hexanoic acid	$C_6H_{10}O_3$	129.1386	129.1371	-0.35	0.48

5.4. Conclusion

The bacterial strains *P. punonensis* and *B. gibsonii* were found to be efficient degraders of long alkyl chain containing DEHP to the extent of 92 to 95%, respectively, at ambient temperature of 30 ^{o}C. The kinetic studies on DEHP degradation showed that removal of DEHP by the bacterium *B. gibsonii* was faster than the *P. punonensis*. Further, it was observed that in both the strains, the DEHP was first degraded to MEHP by the bacterial esterase and then it was converted to MBP through β-oxidation. Finally, the MBP was transformed into PA and then to PCA under aerobic conditions. The PCA is finally cleaved into CO_2 and H_2O via 4-oxo-hexanoic acid. Both *B. gibsonii* and *P. punonensis* strains exhibited their potential use in bioremediation of phthalate contaminated sites.

CHAPTER 6

CHAPTER 6
GENERAL DISCUSSION

The search for novel lipase enzyme with industrial and environmental applications is drawing increasing attention of researchers around the world. Lipase production by extremophilic bacteria under different environmental conditions has held a unique position in search of new kind of useful techniques for bioremediation. Microbial lipase shows lipolytic reactions at the lipid-water interface where lipolytic substrates usually form an equilibrium between monomeric, micellar, and emulsified states. Biotechnological potential of lipase is relying on the ability to catalyze not only the hydrolysis of a given triglyceride, but also its synthesis from glycerol and fatty acids. The main application fields for lipases include detergents, dairy, diagnostics, oil processing, and biotransformation's (Jaeger et al., 1997). Psychrophilic bacteria generally produce a variety of lipase especially by the genus *Pseudomonas* (Rajmohan et al., 2002). Most of the lipases produced by *P. fluorescens* belong to lipase subfamily I.1, I.2, or I.3. Among this group, lipases from subfamily I.3 have comparably larger molecular masses (50 kDa or above) and do not need additional gene products for correct folding (Rosenau and Jaeger 2000). Although both the biochemical and structural properties of lipases from subfamilies I.1 and I.2 have been well-studied, few lipases from subfamily I.3 have been characterized and found to be applicable in enzymatic industry. Even fewer of these enzymes have been found to be useful for enzymatic reactions at industrial scales (Lee et al., 1993, Rashid et al., 2001).

Halophilic microorganisms are salt-loving extremophilic organisms that can often adapt to a broad salt concentration range by maintaining a proper osmotic pressure in their cytoplasm (Detkova et al., 2007). Hypersaline conditions favour protein aggregation and collapse, interfere with the electrostatic interactions between protein residues, and are responsible for a general decrease in the availability of water molecules (Zaccai, 1990).

Halophilic proteins, rather than being unfolded by these conditions, are distinguished by maintaining soluble and active conformations in an environment generally detrimental to other proteins (Siglioccolo et al., 2011). For this reason, several lipolytic enzymes active and stable in extreme conditions of salinity have been recently characterized (Rohban et al., 2009).

The present study is an attempt to explore the kinetic and thermodynamic study of microbial lipase and its role in DEHP degradation. The present study is also an attempt to explore the potential use of microbial lipase under diverse environmental conditions and their bioremediation applications. The *Pseudomonas* strain isolated from psychrophilic environment and *Bacillus* strain isolated from halotolerant environment produce extracellular lipase under different physico-chemical environmental conditions such as, temperature, pH, salinity, different organic solvents, bio surfactants and enzyme inhibitors. The potential application of extracellular lipase from extremophilic microbial strains were capable to degrade phthalate esters.

The evolutionary characteristics of bacteria and their morphological structure, phylogeny and population help us to better understand the adaptability of extremophilic bacteria. The bacteria isolated from soil showed psychrophilic and bacteria isolated from water exhibited halotolerant character when grown under different temperature regime. The macroscopic features of psychrophlic bacteria strain grown on NA showed moderate growth, a yellow color colony with velvet appearance. Psychrophilic strain showed maximum growth on King's B medium while, halotolerant strain showed highest growth on NA medium. Baum et al. (2009) reported the similar growth observation for *Pseudomonas* strains. Macroscopic observation of isolated bacterial strains was found similar with the findings of Rheims et al., (1999) and Kusuma et al., (2014). The shape and size of bacteria was studied under light microscope and Scanning Electron Microscope. The microscopic study revealed the

approx. length of *P. punonensis* was 3.6 µm and 0.7 µm width whereas, the length and width of *B. gibsonii* was observed as 4.64 µm and 0.80 µm respectively.

Molecular identification of isolated *Pseudomonas* strains, using 16S rRNA with universal primers showed that *Pseudomonas* strain (accession no.MH393314) showed 99% similarity to *Pseudomonas punonensis* with the nearest strain *Pseudomonas punonensis* strain LMT03. Whereas, halotolerant *Bacillus* (accession no. MK835660) was similar with *Bacillus* sp. closet to *gibsonii.*

Bacteria are a good source of extracellular lipase production (Javed et al., 2018, Gupta et al., 2004). Lotti et al. (1998) described the expression of lipase in the presence of lipid source as an oil and carbon source. The microbial lipase are generally produced by inducers such as triacylglycerols, fatty acids, hydrolysable esters, tweens, bile salts and glycerol (Dharmsthiti et al., 1998, Shirazi et al., 1998, Rathi et al., 2001 and Ghosh et al. 1996). Ghost et al. (1996) found that different types of nitrogen sources also influence the lipase production. Generally, organic nitrogen sources such as peptone and yeast are preferred for lipase production by *Bacillus* sp. (*Bacillus* strain A30-1, *B. alcalophilus, B. licheniformis* strain H1) and various *Pseudomonads* sp.(viz. *Pseudomonas* sp., *P. fragi, P. fluorescens* BW 96CC), *Staphylococcus haemolyticus* (Wang et al., 1995, Pabai et al., 1996, Ghanem et al., 2000, Lanser et al., 2002 and Sharma et al., 2002).

In the present study, biochemical characteristics of *Pseudomonas* strain and *Bacillus* strain revealed that both the strains are capable to produce extracellular lipase enzyme. However, amylase, protease and xylanase were negative for both the strains.

Previous studies have suggested that *Achromobacter, Alcaligenes, Arthrobacter, Bacillus, burkholderia, Chromobacterium* and *Pseudomonas* are important strains for lipase production. Of these, the lipases from *Pseudomonas* bacteria are widely used for a variety

of biotechnological applications (Jaeger et al. 1994; Pandey et al. 1999; Beisson et al. 2000).

Extracellular lipase was characterized by purified ammonium sulphate precipitation method. The crude lipase from *P. punonensis* was partially purified by using ammonium sulphate precipitation (70% saturation) at 4°C for an overnight dialysis process. The resulted lipase showed an increase in the specific activity of enzyme (149.5 U/mg), indicating the enzyme was concentrated by 1.40-fold. The reports are supported by the findings of Jain et al. (2017) for *Pseudomonas proteolytica* (GBPI_Hb61). Whereas, *B. gibsonii* lipase showed an increase in the specific activity of enzyme (186.3 U/mg), indicating the enzyme was concentrated by 1.36-fold. Kumar et al. (2012), reported that lipase was purified by ammonium sulphate method and specific activity was obtained as 3525.6 U/mg in *B. pumilus* RK31.

Physiological parameters such as pH, temperature, incubation period also play an important role in influencing production of lipase by different microorganisms. Sugihara et al. (1991) reported that bacteria prefer pH around 7.0 for their optimum growth for lipase production. Generally, bacterial lipase has neutral pH or alkaline pH optima with some exceptions as *P. fluorescens* SIK W1 lipase, which has an acidic optimum at pH 4.8. Some thermotolerant bacteria such as *B. stearothermophilus* SB-1, *B. atrophaeus* SB-2 and *B. licheniformis* SB-3 are active over a broad pH range from 3–12 (Bradoo et al., 1999). Bacterial lipases possess stability over a wide range, from pH 4.0-11.0 (Kojima et al., 1994; Wang et al,. 1995; Khyami-Horani, 1996; Dong et al., 1999).

Previous studies on optimum temperature for lipase production revealed that bacterial lipase have temperature optima between 30-60°C (Wang et al., 1995; Dharmsthiti et al., 1998; Litthauer et al., 2002). However, some reports are also available which strongly favour the lipase production in both lower and higher ranges (Lee et al., 1999; Oh et

al., 1999; Sunna et al., 2002). In the present study increase in the enzyme activity of halotolerant *B. gibsonii* with rising temperature, the lipase activity was stimulated until the temperature reached 60°C. Whereas, imposition of low temperature on the psychrotolerant strain *P. punonensis* could not elicit similar response and exhibited maximum activity at 30°C.

Bacterial lipase activity in different organic solvents is desirable for synthesis reaction. Schmidt- Dannert et al. (1994) reported that organic solvents generally enhance the lipase activity with few exceptions of stimulation or inhibition. Acetone, ethanol and methanol enhanced the lipase activity of *B. thermocatenulatus* whereas acetone was inhibitory for *P. aeruginosa* YS-7 lipase and hexane for *Bacillus* sp. lipase (Sugihara et al., 1991). Lipase from *A. calcoaceticus* LP009 was highly unstable with various organic solvents (Dharmsthiti et al., 1998). In our study, the relative lipase activity was stimulated by the presence of 2-propanol, acetone for *P. punonensis* by more than 100% and 133.91% respectively. Whereas, *B. gibsonii* showed enhanced activity when 2-propanol was used as an organic solvent.

Lipase activity does not require any co-factor, but divalent cations such as calcium often stimulate lipase activity (Gupta et al., 2004). It is due to the formation of calcium salts of long-chain fatty acids (Macrae and Hammond 1985; Godtfredsen 1990). In the present study calcium enhance the activity of both *P. punonensis* and *B.gibsonii* extracellular lipase by 138 and 106% respectively. Calcium-stimulated lipases have been reported in the case of *B. subtilis* 168 (Lesuisse et al., 1993), *B. thermoleovorans* ID-1 (Lee et al., 1999), *P. aeruginosa* EF2 (Gilbert et al., 1991b), *S. aureus* 226 (Muraoka et al., 1982), *S. hyicus* (Van Oort et al., 1989), *C. viscosum* (Sugiura et al., 1974) and *Acinetobacter* sp. RAG-1 (Snellman et al., 2002). In contrast, the lipase from *P. aeruginosa* 10145 (Finkelstein et al., 1970) is inhibited by the presence of calcium ions. Further, lipase activity

is in general inhibited drastically by heavy metals like Co^{2+}, $Ni^{2+}$and slightly inhibited by Zn^{2+} and Mg^{2+} (Patkar and Bjorkling 1994). However, the role of Al^{3+} was found to stimulate the lipase activity by 121 and 153% in *P.punonensis* and *B. gibsonii* respectively. For the study of structural and mechanistic properties of lipase some inhibitors were used. This research is also helpful for pharmacological purposes. Inhibitor study is beneficial in designing of drugs for the treatment of obesity and acne problem. Enzyme inhibitors do not act directly at the active site but inhibit lipase activity by changing the conformation of lipase or interfacial properties. Bio surfactants and some proteins were used as inhibitors to study the efficacy of that compound (Iizumi et al., 1990; Patkar and Bjorkling 1994; Gargouri et al., 1984; Bezborodov et al., 1985). However, some surfactants activate the enzyme and this effect could be reversible or irreversible. Lipases belong to the class of serine hydrolase with the catalytic triad as Ser-His-Asp/Glu. Therefore, serine inhibitors are potential irreversible active-site lipase inhibitors such as phenylmethylsulfonyl fluoride (PMSF). In contrast, the lipase from *A. calcoaceticus* LP009 was not inhibited by PMSF (Dharmsthiti et al., 1998). The same trend was observed by the isolated extremophilic bacterial lipase which activity was enhanced by 135 and 104 % for psychrotolerant and halotolerant strains respectively.

However, the affinity of this compound for the enzyme is not high enough, compared with the substrate, and hence it is difficult to obtain useful information from these analogues. Further, EDTA does not affect the activity of most lipases (Gilbert et al., 1991b; Sugihara et al., 1991; Kojima et al., 1994). However, it is inhibitory to lipases from *P. aeruginosa* 10145 (Finkelstein et al., 1970), *Pseudomonas* sp. nov. 109 (Ihara et al., 1991), *Bacillus* sp. THL027 (Dharmsthiti and Luchai 1999) and *A. calcoaceticus* LP009 (Dharmsthiti et al., 1998). EDTA shows inhibitory effect in *B gibsonii* whereas, it enhanced the enzyme activity in *P.punonensis* lipase.

The kinetic constant was calculated from Michaelis–Menten saturation kinetics by using at different concentrations of pNPP (0.1–1.5 mM) as substrate under different temperature conditions (10, 30, 60°C). Maximum velocity was obtained at 30°C (86.22 U/mL) and 60°C (158.73 U/mL) for *P. punonensis* and *B. gibsonii* respectively. The catalytic efficiency constant (kcat/Km) at 30 and 60°C was found as 1073.12 and 913.07, denoting the highest frequency of enzyme–substrate encounter at 30 and 60°C for *P. punonensis* and *B. gibsonii* respectively.

Thermal stability of extracellular lipase from psychrotolerant in terms of maximum stability of enzyme was found at 30°C after 120 min of incubation. However, it was estimated high at 60°C in halotolerant strain after 120 min of incubation. Temperature above 60°C showed a rapid decrease in residual activity of enzyme. Previous study showed that *B. thermoleovorans* ID-1A and *Bacillus* strain lipase was able to retain about 75 and 50% of its activity at 60°C respectively (Kim 1994).

Thermal inactivation of enzyme was studied by measuring the inactivation parameters in terms of inactivation energy, Gibbs free energy, enthalpy, entropy and half-life of enzyme. The inactivation energy was calculated from Arrhenius plot as 56.89 kJ/mol and 66.98 kJ/mol for psychrotolerant and halotolerant strains respectively. The obtained data from inactivation parameters suggested that both lipases possess different characteristics by inactivation temperature and energy. Extracellular lip**as**e from *P. punonensis* showed maximum stability at 30°C whereas, *B. gibsonii* lipase showed maximum stability at 60°C. The results suggested that the inactivation of enzyme was spontaneous and the thermal stability of the enzyme declined with increase in the temperature.

Thermal inactivation of lipase was further confirmed by studying the changes in secondary structure of lipase by Circular dichroism spectra. The result obtained from far- UV CD spectra of lipase at room temperature (30°C) showed the changes at 208 and 222 nm which

denoted the change in α helix of protein during unfolding of *P. punonensis* protein. The intensity of negative peaks at 208 and 222 nm initially increased with rise in temperature up to 50°C. Subsequent rise in the temperature from 70 to 90°C showed decline as well as shift in both the peaks, indicating deformation in the α-helix of the protein structure. High temperature leads to irreversible inactivation of the enzyme due to deformation in the protein structure and loss of catalytic site. Batumalaie (2018) has also reported that the secondary structure of lipase from *Acinetobacter haemolyticus* is denatured due to high temperature treatment. On the other hand, when *B. gibsonii* lipase undergo temperature treatment from 30-90°C, the spectra showed that at peaks obtained at 208 and 222 nm move towards their original position (untreated) after 120 min of incubation. The movement of negative peaks from treated to untreated supports the reversibility of protein secondary structure. High temperature above 60°C leads to reversibility of protein secondary structure for *B. gibsonii* lipase.

The alkaline lipase from extremophilic bacterial strains were used for environmental evaluation to degrade phthalate esters. Di-(2-ethylhexyl) phthalate (DEHP) is one of the most extensively used plasticizer in plastic products due to their flexibility, strength, temperature tolerance and stability (El-Shinnawy, 2016). The DEHP substrate utilization test for *P. punonensis* and *B. gibsonii* confirmed that both the bacterial strains could use DEHP as a sole carbon source. The result of effect of pH and temperature on DEHP degradation showed that pH might not influence the degradation of phthalate whereas, temperature showed important role in the degradation process. Under the acidic conditions, both microbial growth and DEHP removal were much lower compared to the neutral or alkaline conditions. This may be due to the enzyme activities for the PAEs degradation are known to be particularly sensitive to the low pH (Fang et al., 2010). The temperature

conditions showed that *P. punonensis* could easily degrade DEHP up to 30°C whereas, *B. gibsonii* showed degradation from 30 to 50°C after 120 hr of incubation.

The initial concentrations of DEHP (100 mg/L, 250 mg/L, 500mg/L, 750 mg/L and 1000 mg/L) were taken to study the effect of substrate concentrations on biodegradation of DEHP. The degradation of DEHP was enhanced with time and was found maximum at 500 ppm after 120 hr as 92.59% and 95.44% for *B. gibsonii* and *P. punonensis* respectively. Whereas, the degradation rate was found 84.05 and 88.03% for *B. gibsonii* and *P. punonensis* for 1000 ppm after 120 hr of incubation. Similar finding was reported by Zhang et al. (2018) for *Bacillus mojavensis* B1811. The biodegradation pathway of DEHP was examined and its metabolic products checked by GC-MS analysis. The first metabolic products were found as MEHP, peak was observed at 277.1443 (m/z) after at 221.0822 (m/z) the second metabolic product i.e. MBP peak was found. MBP degraded as PA and peak was found at 165.0109 (m/z) and the last intermediated product was detected as 4-oxo-hexanoic acid at 129.1371 (m/z). The final degraded product was generated as CO_2 and H_2O via 4-oxo-hexanoic acid. It was observed that Both *B. gibsonii* and *P. punonensis* strains were capable to degrade DEHP as 95 and 92 % respectively after 5 days of incubation. Some previous studies suggested that *Acinetobacter* sp. SN13 and *Burkholderia pyrrocinia B1213* are also capable to degrade DEHP by 90 and 98% respectively (Xu et al., 2017, Li et al., 2019).

CHAPTER 7

CHAPTER 7
CONCLUSION

The aim of the present study was to assess the production and optimization of extracellular lipase by two different extremophilic bacterial strains and the activity of enzyme under different environmental and physico-chemical conditions with their potential applications in environmental bioremediation. Based on 16S rRNA molecular analysis with universal primers, bacterial isolates were identified as psychrotolerant *Pseudomonas punonensis* (NCBI accession no. MH393314) isolated from agricultural soil of dry temperate region of Leh, Ladakh, India and halotolerant *Bacillus gibsonii* (NCBI accession no. MK 835660) isolated from water collected from Dwaraka Gujrat, India. They also exhibited characteristic features of psychrotolerance and halotolerance when grown under different temperature regimes respcetively. Both the strains grew well and produced extracellular lipase under different environmental and physico- chemical conditions such as temperature, pH, salinity, metal ions, inhibitors, organic solvents and surfactants. The crude lipase from extremophilic bacteria was produced high in the presence of olive oil in production media.

Result on lipase at different conditions revealed that extracellular lipase from *P. punonensis* exhibited maximum activity in the pH range of 7.0-9.0 at 30°C, whereas, *B. gibsonii* lipase showed maximum activity at 60°C at 9.0 pH. Thus, it was concluded that both strains produced extracellular lipase which showed maximum activity at alkaline pH. The result on salt tolerance of *B. gibsonii* lipase revealed that concentration of 600 mM NaCl was required for production of extracellular lipase enzyme. The result of partial purification of lipase from both bacterial strains revealed that ammonium sulphate purification rate was high in *P. punonensis* as compare to *B. gibsonii*. Total enzyme activity was found as 1729.8 U in *P. punonensis* and 1695.78 U in *B. gibsonii*.

The study on the effect of metal ions exhibited that maximum enzyme activity in *P. punonensis* was stimulated by accompanied Ca^{+2} and Al^{+3}, while with some other metals such as Ba^{+2} , Mg^{+2} and K also enhanced the enzyme activity. However, *B. gibsonii* lipase activity was enhanced by only Al^{+3}, whereas rest of the metal ions did not affect the enzyme activity. Hence, it was concluded that Al^{+3} could potentially be used to enhance the lipase activity. The PMSF, serine group specific protein inhibitor stimulated the lipase activity by 172 and 103 % in *P. punonensis* and *B. gibsonii* respectively, when compared with control (100%). EDTA – a known metal chelator stimulated the lipase activity by 172 % and 135% in psychrotolerant and halotolerant strains respectively. Result on lipase activity in the presence of organic solvents and surfactants revealed that SDS stimulated the enzyme activity in case of both bacterial lipases and acetone and 2-propanol could also enhance the lipase.

Thermal characteristic is also an important parameter because temperature plays a major role in the activation or inactivation of enzyme which affects enzyme efficacy during its application. The result of thermal stability of enzyme exhibited that enzyme from *P. punonensis* was stable up to 30°C and beyond this temperature enzyme was rapidly inactivated. However, *B. gibsonii* lipase was found to be thermostable at 60°C and further increase in temperature induced loss of activity.

The thermostability of enzyme was also confirmed by its changes in the secondary structure of the enzyme, which was detected in CD spectra of enzyme, which revealed that temperature treatment changed the secondary structure of psychrotolerant lipase protein which was irreversible. Whereas, when halotolerant lipase treated at high temperature between 60-80°C, the native secondary structure of enzyme was retained in its original form after 60 min of treatment. Thus, it was concluded that secondary structure of lipase from *B.*

gibsonii lipase was reversible.

The search of a novel bacterial enzyme for biodegradation of phthalic acid esters were performed by using isolated bacteria. Both the bacterial strains were capable of degrading DEHP, using their metabolic pathways. The biodegradation kinetics of DEHP revealed that both the bacterial strains could degrade the DEHP after 5 days of incubation.The result of GC-MS/MS revealed that DEHP was degraded into simpler metabolites by bacterial enzymes. The first intermediate product was MEHP, which was further degraded into MBP and PA. The final product was found to be 4-oxo-hexanoic acid which may be further metabolized into CO_2 and H_2O.

The highlights of present study are as follows-

1. *P. punonensis* strain is a psychrotolerant, whereas *B. gibsonii* halotolerant in nature.
2. Both the isolated bacteria are good producers of lipase enzyme.
3. At low temperature, psychrotolerant *P. punonensis* showed optimum growth at 20°C whereas, *B. gibsonii* exhibited optimum growth at 30°C.
4. *B. gibsonii* was more tolerant to high concentration of salt than the psychrotolerant *P. punonensis*.
5. Maximum lipase activity by *P. punonensis* was obtained at 30°C and pH 7.0. Whereas, *B. gibsonii* lipase showed its maximum activity at 60°C and pH 9.0. Lipase activity was found to be maximum at alkaline pH 11.0.
6. The effect of physico-chemical conditions (temperature, pH, metals ions, surfactants, inhibitors and organic solvents) exhibited the sensitivity of lipase.
7. Study of kinetic and thermal properties of both lipases showed different characteristics. Lipase from *P. punonensis* showed maximum activity and thermal stability at 30°C, whereas, *B. gibsonii* lipase showed maximum activity and thermal stability at 60°C.

8. The CD spectra in far UV region (210-240 nm) suggested that *P. punonensis* lipase was heat labile and its effect was irreversible in nature after temperature treatment. However, *B. gibsonii* lipase showed reversibility in heat induced changes in the secondary structure of protein after 60 min.
9. The degradation of DEHP was enhanced with time and was found to be maximum at 500 ppm after 120 h. The DEHP degradation was about 92.59 % and 95.44 % for *B. gibsonii* and *P. punonensis* strains, respectively.
10. The biodegradation pathway includes MEHP, MBP, PCA and 4-oxo-hexanoic metabolites as intermediates of DEHP degradation.

SUMMARY

SUMMARY

Lipases from microbial source have valuable industrial applications in enhancing cheese flavour, organic synthesis, oil structural modification, and biodegradation of plasticizers and phthalic acid esters (PAEs). Due to their multifunctionality and various properties, lipases have attracted great interest in biodegradation of plasticizers and PAEs and many research efforts have been made to find lipases suitable for different phthalic esters degradation.

The production of lipase from bacteria may depend upon the nutrient availability, physical and environmental conditions. However, the production of microbial lipase in extreme environmental conditions could be a new approach and may be an area of interest as the production of bioactive molecules may get modified and enhanced.

The present study was an attempt to explore the bacterial lipase from two different extremophlic strains isolated from two diverse climatic zone. *Pseudomonas punonensis* isolated from cold-environment soil was psychrotolerant whereas, *Bacillus gibsonii* isolated from saline water halotolerant in nature. Both the isolated strains produced lipase, which was further optimized in different environmental and physico-chemical conditions of pH, temperature, metal ions, inhibitors and surfactants. Further, its kinetic and thermal properties were also studied for environmental application. Thermal activation and inactivation of enzyme helps in the implementation of its environmental aspects. The result of thermal inactivation of *B. gibsonii* was further confirmed by UV-CD spectra. The change in secondary structure of protein was found as reversible after high temperature treatment. Whereas, *P. punonensis* CD spectra of enzyme was found as irreversible after temperature treatment.

Biodegradation of DEHP (di (2- ethylhexyl) phthalate) was examined by isolated bacterial

strains. DEHP utilization and degradation were estimated by mass spectrophotometer. The residual concentration of DEHP and intermediated products formed during biodegradation were detected by GC-MS/MS. However, several studies on phthalate degradation were conducted by different types of bacteria but we have chosen DEHP due to their complexity and worldwide use as plasticizer.

The findings of the study are summarized as below-

- Two extremophilic bacterial strains were isolated from two different climatic zones of India, one from agricultural soil of dry temperate region of Leh, Ladakh, India and another from saline water from Dwraka Gujrat, India.
- The microscopic morphological characters were used for the identification of bacterial strains and it was concluded that one strain belong to genus *Pseudomonas* other to *Bacillus*.
- Based on 16 S rRNA gene sequencing and comparison with NCBI GenBank database, strains were identified as psychrotolerant *P. punonensis* (accession no. MH 393314) and halotolerant *B. gibsonii* (accession no. MK835660).
- The present work revealed that the psychrotolerant *P. punonensis* has potential to grow at low temperature (15°C) with growth optima at 25°C and alkaline pH 7.0. The halotolerant *B. gibsonii* showed optimum growth between 30-35°C and alkaline pH 7.0-8.0.
- Further, it was observed that mesophilic *B. gibsonii* was able to tolerate NaCl concentrations (upto 1200 mM) and showed optimum growth at 600 mM salt concentration. Hence, *B. gibsonii* was found to be halotolerant in nature.
- Biochemical characteristics of both extremophilic strains revealed that both could efficiently produce extracellular lipase enzymes.

- Screening of lipase enzyme was done by using tributyrin agar method and production was enhanced by adding olive oil.
- Lipase was purified by ammonium sulphate purification method with purification fold 1.40 in *P. punonensis* and 1.36 purification fold in *B. gibsonii* than crude enzyme.
- Optimization of lipase activity was observed under different physico-chemical environmental conditions such as pH, temperature, metal ions, enzyme inhibitors, surfactants and organic solvents.
- Optimum pH and temperature were found as 7.0 and 30°C for *P. punonensis* lipase, whereas, it was found 9.0 at 60°C for *B. gibsonii* lipase.
- Among different metal ions Ca^{+2} and Al^{+3} was found as enhancers for both extremophilic lipases activity whereas, Mn^{+2}, Ba^{+2}, Co^{+2} inhibited the activity of enzyme.
- Among different inhibitors, PMSF, DTT and β-2 mercaptoethanol, PMSF enhanced the activity of lipase isolated from both bacterial strains.
- Among different surfactants and organic solvents such as Tween 20, Tween 80, CTAB, EDTA, SDS, acetone, 2-butanol, ethanol, methanol and 2-propanol, lipase activity was enhanced by EDTA by 172 % in *P. punonensis* whereas, SDS slightly enhanced lipase activity for *B. gibsonii.* It was found that *P. punonensis* lipase was enhanced by acetone whereas, ethanol and 2- propanol enhanced the *B. gibsonii* lipase activity.
- Kinetic study was examined by using p-NPP as substrate at different concentrations ranging from 0.1-1.0 mM at different temperature range (10, 30, 60°C). Kinetic constant such as Michaelis-Menten constant (Km) and Vmax were calculated by Lineweaver-Burk plot.

- Kinetic study of both types of lipase revealed that maximum velocity and Michaelis-Menten constant (Km) was found at 30°C as 86.22 U/mL and 0.163 mM for *P. punonensis* lipase. Whereas, *B. gibsonii* lipase showed its maximum velocity158.72 U/mL at 0.53 mM substrate concentration at 60°C.
- The catalytic efficiency and turn over number of lipases revealed that enzyme activity substrate enzyme binding complex was found high at 30°C in *P. punonensis*. However, it was estimated high at 60°C for *B. gibsonii* lipase.
- The activation energy was calculated as 27.74 kJ/mol for *P.punonensis* and 45.28 kJ/mol for *B. gibsonii* lipase from Arrhenius plot.
- Thermal stability of enzyme was estimated by calculating residual activity at different temperature *P. punonensis* and *B. gibsonii* lipase. It was found that *P. punonensis* lipase was found maximum stable at 30°C whereas, at 60°C, *B. gibsonii* lipase achieved its maximum stability.
- Thermal inactivation was also studied by calculating inactivation energy, deactivation constant, Desidual, Gibbs free energy, enthalpy, entropy and half-life of enzyme. The result of thermal inactivation of enzyme suggested that 56.89 kJ/mol deactivation energy was needed by *P. punonensis*. However, 66.98 kJ/mol energy is needed to inactivate *B. gibsonii* lipase.
- The effect of temperature on protein secondary structure were determine by far UV-CD spectra. The change in α-helix of protein was observed by increasing temperature above optimum. The change in secondary structure were found irreversible for *P. punonensis*, whereas, it was found reversible after 60 min in *B. gibsonii*.

- Both the isolated extremophilic strains were used to degrade phallic acid esters. DEHP was chosen to study the potential use of isolated strains in environment pollution management.

REFERENCES

REFERENCES

Abdel-Fattah, Y.R. and Gaballa, A.A. (2008). Identification and over-expression of a thermostable lipase from *Geobacillus thermoleovorans* Toshki in *Escherichia coli*. *Microbiological research*, 163(1), 13-20.

Abdulkadir, M., & Waliyu, S. (2012). Screening and isolation of the soil bacteria for ability to produce antibiotics. *European Journal of Applied Sciences*, *4*(5), 211-215.

Abel, H., Marie, D., Nathalie, R., Danielle, D., Louis, S., Louis, C. (2000). Purification and characterization of an extracellular lipase from a thermophilic *Rhizopus oryzae* strain isolated from palm fruit. *Enzyme and Microbial Technology*, 26, 421–430

Abol Fotouh, D. M., Bayoumi, R. A., & Hassan, M. A. (2016). Production of thermoalkaliphilic lipase from Geobacillus thermoleovorans DA2 and application in leather industry. *Enzyme Research*, 2016.

Achal, V., Mukherjee, A., & Reddy, M. S. (2010). Characterization of two urease-producing and calcifying *Bacillus* spp. isolated from cement. *Journal of microbiology and biotechnology*, *20*(11), 1571-1576.

Acharya, S., & Chaudhary, A. (2012). Bioprospecting thermophiles for cellulase production: a review. *Brazilian Journal of Microbiology*, *43*(3), 844-856.

Adams, D. M., & Brawley, T. G. (1981). Factors influencing the heat resistance of a heat-resistant lipase of *Pseudomonas*. *Journal of Food Science*, *46*(3), 673-676.

Aderiye, B., & Sulaimon, A. (2017). Optimization and lipase production of *Lysinibacillus sphaericus* in domestic oil rich waste water. *Biotechnology Journal International*, 1-12.

Agobo, K. U., Arazu, V. A., Uzo, K., & Igwe, C. N. (2017). Microbial lipases: a prospect for biotechnological industrial catalysis for green products: a review. *Ferment Technol*, 6(144), 2.

Ahn, J., Kim, Y., Min, J., Lee, J. (2006). Accelerated degradation of dipentyl phthalate by *Fusarium oxysporum* f. sp. pisi cutinase and toxicity evaluation of its degradation products using bioluminescent bacteria. *Current Microbiology*, 52 (5), 340e344.

Ahuactzin-Pérez, M., Tlecuitl-Beristain, S., García-Dávila, J., González-Pérez, M., GutiérrezRuíz, M.C., Sánchez, C. (2016). Degradation of di(2-ethyl hexyl) phthalate by *Fusarium culmorum*: kinetics, enzymatic activities and biodegradation pathway based on quantum chemical modeling pathway based on quantum chemical modeling. *Science of Total Environment*, 566–567, 1186–1193

Ahuja SK, Ferreira GM, Moreira AR. (2004). Utilization of enzyme for environmental application, *Critical Review in Biotechnology*, Vol. 24, 125-154.

Alkan H, Baysal Z, Uyar F, Dogru M. (2007). Production of lipase by a newly isolated *Bacillus coagulans* under solid-state fermentation using melon waste, *Applied Biochemistry and Biotechnology*, Vol. 136, 183-192.

Aloulou A, Rodriguez JA, Frédéric C. (2006). Exploring the specific features of interfacial enzymology based on lipase studies. *BBA (Mol Cell Biol Lipids)*,1761:995–1013.

Ameri, A., Shakibaie, M., Soleimani-Kermani, M., Faramarzi, M. A., Doostmohammadi, M., & Forootanfar, H. (2019). Overproduction of thermoalkalophilic lipase secreted by *Bacillus atrophaeus* FSHM2 using UV-induced mutagenesis and statistical optimization of medium components. *Preparative Biochemistry and Biotechnology*, *49*(2), 184-191.

Andree H, Muller WR, Schmid RD. (1980). Lipases as detergent components, *Journal of Applied Biochemistry*, Vol. 218-219.

Aravindan, R., Anbumathi, P and Viruthagiri, T. (2007). Lipase applications in food industry, *Indian Journal of Biotechnology*, 6: 141-158.

Archibald FS. (1992). A new assay for lignin-type peroxidases employing the dye azure B. *Applied and Environmental Microbiology*, 58(9): 3110–3116.

Arpigny JL, Jaeger KE. (1999). Bacterial lipolytic enzymes: classification and properties, *Biochemical Journal*, Vol. 343, 177-183.

Arpigny JL, Lamotte J, Gerday C. (1997). Molecular adaptation to cold of an Antarctic bacterial lipase. *Journal of Molecular and Catalysis B: Enzymatic,* 3: 29-35.

Arroyo M and Sinisterra JV. (1995). Influence of chiral corvones on selectivity of pure lipase-B from *Candida antarctica*. *Biotechnology Letter,* 17: 525-530.

Babu, I.S and Rao, G.H. (2007). Lipase production by *Yarrowia lipolytica* NCIM 3589 in solid state fermentation using mixed substrate. *Research Journal of Microbiology,* 2: 469-474.

Baharum, S. N., Salleh, A. B., Razak, C. N. A., Basri, M., Rahman, M. B. A., & Rahman, R. N. Z. R. A. (2003). Organic solvent tolerant lipase by *Pseudomonas* sp. strain S5: stability of enzyme in organic solvent and physical factors effecting its production. *Annals of microbiology*, 53(1), 75-84.

Bajaj, A.; Lohan, P.; Jha, P. N.; Mehrotra, R (2010). Biodiesel production through lipase catalyzed transesterification: an overview. *Journal of Molecular Catalysis B: Enzymatic*, 62, (1), 9-14.

Balaji L, Jayaraman G. (2014). Metal ion activated lipase from halotolerant *Bacillus* sp. VITL8 displays broader operational range. *International Journal of Biological Macromolecules*. 67: 380–386

Balaji, L., Chittoor, J. T., & Jayaraman, G. (2020). Optimization of extracellular lipase production by halotolerant *Bacillus* sp. VITL8 using factorial design and applicability of enzyme in pretreatment of food industry effluents. *Preparative biochemistry & biotechnology*, *50*(7), 708-716.

Balan, A., Ibrahim, D., Abdul Rahim, R., Rashid, A. and Azzahra, F. (2012). Purification and characterization of a thermostable lipase from *Geobacillus thermodenitrificans* IBRL-nra. *Enzyme research*, *2012*.

Bancerz, R., Ginalska, G., Fiedurek, J and Gromada, A. (2005). Cultivation conditions and properties of extracellular crude lipase from the psychrotrophic fungus *Penicillium chrysogenum* 9'. *Journal of Industrial Microbiology and Biotechnology*, 32: 253–260.

Barbaro SE, Trevors JT, Inniss WE. (2001). Effects of low temperature, cold shock and various carbon sources on esterase and lipase activities and exopolysaccharide production by a psychrotrophic Acinetobacter sp., *Canadian Journal of Microbiology*, Vol. 47, 194-205.

Barrios-González, J. (2012). Solid-state fermentation: physiology of solid medium, its molecular basis and applications. *Process Biochemistry*, 47 (2), 175-185.

Barros, M.; Fleuri, L.; Macedo, G. (2010). Seed lipases: sources, applications and properties-a review. *Brazilian Journal of Chemical Engineering*, 27, (1), 15-29.

Batumalaie, K., Khalili, E., Mahat, N. A., Huyop, F., & Wahab, R. A. (2018). Biophysical characterization of a recombinant lipase KV1 from *Acinetobacter haemolyticus* in relation to pH and temperature. *Biochimie*, 152, 198-210.

Bayoumi, R. A., Atta, H. M., & El-Sehrawy, M. H. (2012). Bioremediation of khormah slaughter house wastes by production of thermoalkalistable Lipase for application in leather industries. *Life Science Journal*, 9(4), 1324-1335.

Behera, A. R., Veluppal, A., & Dutta, K. (2019). Optimization of physical parameters for enhanced production of lipase from *Staphylococcus hominis* using response surface methodology. *Environmental Science and Pollution Research*, *26*(33), 34277-34284.

Benjamin, S., Kamimura, N., Takahashi, K., Masai, E. (2016). *Achromobacter denitrificans* SP1 efficiently utilizes 16 phthalate diesters and their downstream products through protocatechuate 3,4-cleavage pathway. *Ecotoxicology and Environmental Safety*, 134, 172e178.

Berglund P and Hutt K. (2000). Biocatalytic synthesis of enantiopure compounds using lipases. In: Patel RN, editor. Stereoselective biocatalysis. New York: Marcel Dekker.

Berglund P. (2001). Controlling lipase enatioselectivity for organic synthesis. *Biomolecular Engineering* IS: 13-22.

Bharathi, D., Rajalakshmi, G., & Komathi, S. (2019). Optimization and production of lipase enzyme from bacterial strains isolated from petrol spilled soil. *Journal of King Saud University-Science*, *31*(4), 898-901.

Bhushan, I., Yadav, A.K and Parshad, R. (2011). Enhancement in the production of lipase from *Arthrobacter* sp. using fed- batch fermentation strategy, *Asiatic Journal of Biotechnology Resources*, 2 (5): 522-534.

Bisht, D., Yadav, S.K and Darmwal N.S. (2012). Enhanced production of extracellular alkaline lipase by an improved strain of *Pseudomonas aeruginosa* MTCC 10,055, *Am. Journal of Applied Sciences*. 9 (2): 158-167.

Bisht, Deepali, Santosh Kumar Yadav, and Nandan Singh Darmwal. (2013). An oxidant and organic solvent tolerant alkaline lipase by *P. aeruginosa* mutant: downstream processing and biochemical characterization." *Brazilian Journal of Microbiology* 44.4: 1305-1314.

Bolhuis AKD, Thomas JR. (2008). Halophilic adaptations of proteins. Protein adaptation in extremophiles. K.S. Siddiqui and T Thomas. New York, Nova Science: 71–104

Booth I. (1985). Regulation of cytoplasmic pH in bacteria. *Microbiological Reviews*, 49: 359–78.

Bornscheuer UT. (2002). Microbial carboxyl esterases: classification, properties and application in biocatalysis, *FEMS Microbiology Reviews*, Vol. 26, No. 1, pp. 73-81.

Bose, A.; Keharia, H. (2013). Production, characterization and applications of organic solvent tolerant lipase by *Pseudomonas aeruginosa* AAU2. *Biocatalysis and Agricultural Biotechnology*, 2 (3), 255-266.

Bradoo S, Saxena RK, Gupta R. (1999). Two acidothermotolerant lipases from new variants of *Bacillus* sp., *World Journal of Microbiology and Biotechnology*, Vol. 15, 87-91.

Brock TD. (1985). Life at high temperatures. Science 230(4722):132–138

Brune AK, Gotz F. (1992). Degradation of lipids by bacterial lipases. In: Winkelman, G. (ed), Microbial degradation of natural products, VCH, Weinhein, 243-266

Bussamara, R., Fuentefria, A.M., de Oliveira, E.S., Broetto, L., Simcikova, M and Valente, A. (2010). Isolation of a lipase secreting yeast for enzyme production in pilot-plant scale batch fermentation. *Bioresource Technology*, 101:268-275.

Buzzini, P., Branda, E., Goretti, M., & Turchetti, B. (2012). Psychrophilic yeasts from worldwide glacial habitats: diversity, adaptation strategies and biotechnological potential. *FEMS microbiology ecology*, *82*(2), 217-241.

Cadirci BH, Yasa I. (2010). An organic solvents tolerant and thermotolerant lipase from 687 *Pseudomonas fluorescens* P21. *Journal of Molecular Catalysis B: Enzymatic*, 64: 155–161688.

Cai, H., Zheng, W., Zheng, P., Wang, S., Tan, H., He, G., & Qu, W. (2015). Human urinary/seminal phthalates or their metabolite levels and semen quality: A meta-analysis. *Environmental research*, *142*, 486-494.

Cai, J., Xie, Y., Song, B., Wang, Y., Zhang, Z and Feng, Y. (2010). *Fervidobacterium changbaicum* Lip1: identification, cloning, and characterization of the thermophilic lipase as a new member of bacterial lipase family V, *Applied Microbiology and Biotechnology*, 89 : 1463-1473.

Carvalho, A.; Fonseca, T. D.; de Mattos, M. C.; de Oliveira, M. D. F.; de Lemos, T. L. G.; Molinari, F.; Romano, D.; Serra, I. (2015). Recent Advances in Lipase-Mediated Preparation of Pharmaceuticals and Their Intermediates. *International Journal of Molecular Sciences*, 16, (12), 29682-29716.

Castilla, A., Panizza, P., Rodríguez, D., Bonino, L., Díaz, P., Irazoqui, G., & Giordano, S. R. (2017). A novel thermophilic and halophilic esterase from *Janibacter* sp. R02, the first member of a new lipase family (Family XVII). *Enzyme and microbial technology*, *98*, 86-95.

Cavicchioli, R., Charlton, T., Ertan, H., Omar, S. M., Siddiqui, K. S., & Williams, T. J. (2011). Biotechnological uses of enzymes from psychrophiles. *Microbial biotechnology*, *4*(4), 449-460.

Cesarini, S.; Pastor, F.; Nielsen, P. M.; Diaz, P. (2015). Moving towards a Competitive Fully Enzymatic Biodiesel Process. Sustainability, 7, (6), 7884-7903.

Chakravorty, D., Parameswaran, S., Dubey, V. K and Patra, S. (2011). In silico characterization of thermostable lipases. *Extremophiles*, 15: 89 - 103.

Chang, B. V., Wang, T. H., & Yuan, S. Y. (2007). Biodegradation of four phthalate esters in sludge. *Chemosphere*, *69*(7), 1116-1123.

Chang, B.V., Yang, C.M., Cheng, C.H., Yuan, S.Y. (2004). Biodegradation of phthalate esters by two bacteria strains. *Chemosphere* 55, 533–538

Chartrain, M., Katz, L., Marcin, C., Thien, M., Smith, S., Fisher, F., Goklen, K., Salmon, P., Brix, T., Price, K and Greasham, R. (1993). Purification and 9characterization of a novel bioconverting lipase from *Pseudomonas aeruginosa* MB 5001. *Enzyme and Microbial Technology*, 15:575–80.

Chen, J., Ishii, T., Shimura, S., Kirimura, S and Usami, S. (1992). Lipase production of *Tricosporon fermentans* WUC12 a newly isolated yeast. *Journal of Fermentation and Bioengineering*, 73: 412- 414.

Cherif, S., Mnif, S., Hadrich, F., Abdelkafi, S and Sayadi, S. (2011). Strategy for improving extracellular lipolytic activates by a novel thermotolerant *Staphylococcus* sp. strain. *Lipids in Health and Disease* 10:209.

Chintalapati, S., Kiran, M. D., & Shivaji, S. (2004). Role of membrane lipid fatty acids in cold adaptation. *Cellular and molecular biology (Noisy-le-Grand, France)*, *50*(5), 631-642.

Choudhary, R.; Jana, A.; Jha, M. (2004). Enzyme technology applications in leather processing. *Indian Journal of chemical technology*, 11, 659-671.

Christakopoulos, P., Tzia, C., Kekos, D and Macris, B.J. (1992). Production and characterization of extracellular lipase from *Calvatia goatee*. *Applied Microbial Biotechnology*, 194-197.

Christianah, A.T. B., Musa, H and Adeyinka, A.T. (2012). Utilization of nutrients for growth and lipase production by some selected lipolytic fungi, New York Science Journal. (10): 136 – 141.

Christopher, L. P., Zambare, V. P., Zambare, A., Kumar, H., & Malek, L. (2015). A thermo-alkaline lipase from a new thermophile *Geobacillus thermodenitrificans* AV-5 with potential application in biodiesel production. *Journal of Chemical Technology & Biotechnology*, *90*(11), 2007-2016.

Clarridge, J. E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical microbiology reviews*, *17*(4), 840-862.

Coenen TMM, Aughton P, Verhagan H. (1997). Safety evaluation of lipase derived from *Rhizopus oryzae*: Summary of toxicological data. *Food and Chemical Toxicology*, 35: 315–22.

Colin, V. L., Baigori, M. D and Pera, L. M. (2010). Effect of environmental conditions on extracellular lipases production and fungal morphology from *Aspergillus niger* MYA 135. *Journal of Basic Microbiology*, 50: 52-58.

Colla, L.M., Ficanha, A.M., Rizzardi, J., Bertolin, T.E., Reinehr, C.O., Costa, J.A.V., (2015). Production and characterization of lipases by two new isolates of Aspergillus through solid-state and submerged fermentation. BioMed Research International.

Collins, T., Roulling, F., Piette, F., Marx, J. C., Feller, G., Gerday, C., & D'Amico, S. (2008). Fundamentals of cold-adapted enzymes. In *Psychrophiles: from biodiversity to biotechnology* ,211-227

Colonia, B.S.O., Woiciechowski, A.L., Malanski, R., Letti, L.A.J., Soccol, C.R., (2019). Pulp improvement of oil palm empty fruit bunches associated to solid-state biopulping and biobleaching with xylanase and lignin peroxidase cocktail produced by *Aspergillus* sp. LPB-5. *Bioresource Technology*, 285, 121361.

Coradi, G.V., Da Visitação, V.L., De Lima, E.A., Saito, L.Y.T., Palmieri, D.A., Takita, M. A., De Lima, V.M.G., (2013). Comparing submerged and solid-state fermentation of agroindustrial residues for the production and characterization of lipase by *Trichoderma harzianum*. *Annals of Microbiology*, 63 (2), 533-540.

Costa, T.M., Hermann, K.L., Garcia-Roman, M., Valle, R.D.C.S.C. and Tavares, L.B.B. (2017). Lipase production by *Aspergillus niger* grown in different agro-industrial wastes by solid-state fermentation. *Brazilian Journal of Chemical Engineering*, 34 (2), 419-427.

Cotter PD, Hill C. (2003). Surviving the Acid Test: Responses of Gram-Positive Bacteria to Low pH. *Microbiology and Molecular Biology Reviews*; 67(3): 429–453.

Dahiya, P., Arora, P., Chaudhury, A., Chand, S., & Dilbaghi, N. (2010). Characterization of an extracellular alkaline lipase from *Pseudomonas mendocina* M-37. *Journal of basic microbiology*, *50*(5), 420-426.

Das, A., Bhattacharya, S., Shivakumar, S., Shakya, S., Sogane, S.S. (2016). Coconut oil induced production of a surfactant-compatible lipase from *Aspergillus tamarii* under submerged fermentation. *Journal of basic microbiology*. 57, 114–120.

Detkova EN, Boltyanskaya YV. (2007). Osmoadaptation of haloalkaliphilic bacteria: role of osmoregulators and their possible practical application. Mikrobiologiia 76(5):581–593.

Dharmsthiti S, Kuhasuntisuk B. (1998). Lipase from *Pseudomonas aeruginosa* LP602: biochemical properties and application for wastewater treatment, *Journal of Industrial Microbiology and Biotechnology*, Vol. 21, 75-80.

Divakar, S and Manohar, B. (2007). Use of lipase in the industrial production of esters. In: Industrial Enzymes. (Eds.), Olaina, J and MacCabe, A.P. Wiley VCH, Verlag, 283-300.

Do, H., Lee, J. H., Kwon, M. H., Song, H. E., An, J. Y., Eom, S. H., & Kim, H. J. (2013). Purification, characterization and preliminary X-ray diffraction analysis of a cold-active lipase (CpsLip) from the psychrophilic bacterium *Colwellia psychrerythraea* 34H. *Acta Crystallographica Section F: Structural Biology and Crystallization Communications*, *69*(8), 920-924.

Dominguez A, Costas M, Longo MA, Sanroman A. (2003). A novel application of solid state culture: Production of lipases by *Yarrowia lipolytica*, *Biotechnology Letter*, Vol. 25,1225-1229.

Dong, H., Gao, S., Han, S.P and Cao, S.G. (1999). Purification and characterization of a *Pseudomonas* sp. lipase and its properties in non-aqueous media. *Biotechnology and Applied Biochemistry*, 30: 251-256.

Dong-Woo, L., You-Seok, K., Ki Jun, K., Byung-Chan, K., HakJong, C., Doo-sik, K., Maggy, T., Yu-ryang, P. (1999). Isolation and characterisation of thermophilic lipase from *Bacillus thermoleovorans* ID-1. *FEMS Microbiology Letters*, 179, 393–400.

Dörmő, N., Bélafi-Bakó, K., Bartha, L., Ehrenstein, U., & Gubicza, L. (2004). Manufacture of an environmental-safe biolubricant from fusel oil by enzymatic esterification in solvent-free system. *Biochemical Engineering Journal*, *21*(3), 229-234.

Doukyu, N and Ogino, H. (2010). Organic solvent tolerant enzymes. *Biochemical Engineering Journal*, 48: 270-282.

Dutra, J. C., da Terzi, S. C., Bevilaqua, J. V., Damaso, M. C., Couri, S., Langone, M. A., & Senna, L. F. (2007). Lipase production in solid-state fermentation monitoring biomass growth of *Aspergillus niger* using digital image processing. In *Biotechnology for Fuels and Chemicals*, 431-443.

Ebrahimpour A, Rahman RNZRA, Basri M, Salleh AB. (2011). High level expression and characterization of a novel thermostable, organic solvent tolerant, 1,3-regioselective lipase from *Geobacillus* sp. strain ARM. *Bioresource Technology*, 102: 6972–6981.

Eggert T, Brockmeier U, Droge MJ, Quax WJ, Jaeger KE. (2003). Extracellular lipases from *Bacillus subtilis*: regulation of gene expression and enzyme activity by amino acid supply and external pH, *FEMS Microbiology Letters*, Vol. 225, 319-24.

Eijkman C. (1901). U¨ ber Enzyme bei bakterien und Schimmelpilzen, Cbl Bakt Parasitenk Infektionskr, Vol. 29, 841-848.

Elibol, O., Yüksel, N., Egilmez, H. R., Arici, S., & Mizrak, B. (1995). A case of primary orbital melanoma treated by local excision. *The British journal of ophthalmology*, *79*(12), 1146.

El-Shinnawy, N.A. (2016). The therapeutic applications of celery oil seed extract on the plasticizer di-(2-ethylhexyl) phthalate toxicity. *Toxicology and Industrial Health*, 31, 355–366.

Emtenani, S., Asoodeh, A., & Emtenani, S. (2013). Molecular cloning of a thermo-alkaliphilic lipase from *Bacillus subtilis* DR8806: expression and biochemical characterization. *Process biochemistry*, *48*(11), 1679-1685.

Ertugrul S, Donmez G, Takaç S. (2007). Isolation of lipase producing *Bacillus* sp. from olive mill wastewater and improving its enzyme activity, *Journal of Hazardous Materials*, Vol. 149,720-724.

Espinosa, E., Sachez, S and Farres, A. (1990). Nutritional factors affecting lipase production by *Rhizopus delemar* CDBB H313. *Biotechnology letters*, 12: 209 -214.

Evans M, Davies JK, Sundqvist G, Figdor D. (2002).Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. *Inter Endodontic* J 2002; 35: 221–228.

Fadiloglu, S and Erkmen, O. (1999). Lipase production by *Rhizopus oryzae* growing on different carbon and nitrogen sources. *Journal of the Science of Food and Agriculture*. 79: 1936-1938.

Feller G, Gerday C. (2003). Psychrophilic enzymes: Hot Topics in Cold Adaption, *Nature Review Microbiology*, 1:200-208.

Feller, G. (2013). Psychrophilic enzymes: from folding to function and biotechnology. *Scientifica*, *2013*.

Fernandes MLM, Saad EB, Meira JA, Ramos LP, Mitchell DA, Krieger N. (2007). Esterification and transesterification reactions catalyzed by addition of fermented solids to organic reaction media, *Journal of Molecular Catalysis B: Enzymatic*, Vol. 44, 8-13.

Fernandez VG, Brieva R, Gotor V. (2006a). Lipases: useful biocatalysts for the preparation of pharmaceuticals, *Journal of Molecular Catalysis B: Enzymatic*, Vol. 40, 111-120.

Fernandez VG, Busto E, Gotor V. (2006b). *Candida antarctica* lipase B: an ideal biocatalyst for the preparation of nitrogenated organic compounds, *Advance Synthesis and Catalysis*, Vol. 348, 797-812.

Ferrato F, Carriere F, Sarda L. (1997). A critical reevaluation of the phenomenon of interfacial activation. *Methods in Enzymology*, 286:327–347

Ferreira L, Carvalho R Gil MH and Dordick JS. (2002). Enzymatic synthesis of inulin-containing hydrogels. *Biomacromolecules*, 3: 333-334.

Fuji T, Tatara T, Minagwa M (1986). Studies on Application of lipolytic enzyme in Detergency I. *Journal of the American Oil Chemists' Society*, 63: 796-799.

Gabriela AM, Maria MSL and Glaucia MP. (2003). Enzymatic synthesis of short chain citronellyl esters by a new lipase from *Rhizopus* sp. *Process Biotechnology* (Electronic journal) 6(1), 3-4.

Gandhi, N. N. (1997). Application of Lipase, Journal of the American Oil Chemists' Society. 74: 621-634.

Ganguly, S.; Nandi, S. (2015). Process optimization of lipase catalyzed synthesis of diesters in a packed bed reactor. *Biochemical Engineering Journal*, 102, 2-5.

Gao, M., Qi, Y., Song, W., & Xu, H. (2016). Effects of di-n-butyl phthalate and di (2-ethylhexyl) phthalate on the growth, photosynthesis, and chlorophyll fluorescence of wheat seedlings. *Chemosphere*, *151*, 76-83.

Ghanem EH, Al-Sayed HA, Saleh KM. (2000). An alkalophilic thermostable lipase produced by new isolate of *Bacillus alcalophilus*, *World Journal of Microbiology and Biotechnology*, Vol. 16, No. 5, 459-464.

Ghasemi Y, Rasoul_Amini S, Kazemi A, Zarrini G, Morowvat MH, Kargar M. (2011). Isolation and characterization of some moderately halophilic bacteria with lipase activity. *Microbiology*, 80(4): 483–487.

Ghosh, P.K., Saxena, R.K., Gupt, R., Yadav, R.P and Davidson, W.S. (1996). Microbial lipases: production and applications. *Science Progress*., 79:119-157.

Gilbert EJ. (1993). *Pseudomonas* lipases: biochemical properties and molecular cloning, *Enzyme and Microbial Technology*, Vol. 15, No. 8, 634−645.

Giuseppin M.L.F. (1984). Effects of dissolved oxygen concentration on lipase production by *Rhizopus delemar*. *Applied Microbiology and Biotechnology*, 20: 161-165.

Godfrey, T and West, S. (1996). The application of enzymes in industry. In: Industrial enzymology, (Eds) Godfrey, T and Reichelt, J 2nd (edn). The Nature Press, New York, p. 512.

GÖKBULUT, A. A., & Arslanoğlu, A. (2013). Purification and biochemical characterization of an extracellular lipase from psychrotolerant *Pseudomonas fluorescens* KE38. *Turkish Journal of Biology*, *37*(5), 538-546.

Golaki, B., Aminzadeh, S., Karkhane, A. A., Yakhchali, B., Farrokh, P., Khaleghinejad, S. H., & Mehrpooyan, S. (2015). Cloning, expression, purification, and characterization of lipase 3646 from thermophilic indigenous *Cohnella* sp. A01. *Protein expression and purification*, *109*, 120-126.

Gopinath, S.C.B., Hilda, A., Lakshmi priya, T and Annadurai, G. (2002). Purification of lipase from *Cunninghamella verticillata* and optimization of enzyme activity using response surface methodology. *World Journal of Microbiology and Biotechnology*, 18: 449-458.

Goswami, D., De, S. and Basu, J.K. (2012). Effects of process variables and additives on mustard oil hydrolysis by porcine pancreas lipase. *Brazilian Journal of Chemical Engineering*, 29(3), 449-460.

Goswami, D., Parmar, S., Vaghela, H., Dhandhukia, P., & Thakker, J. N. (2015). Describing *Paenibacillus mucilaginosus* strain N3 as an efficient plant growth promoting rhizobacteria (PGPR). *Cogent Food & Agriculture*, *1*(1), 1000714.

Gotz F, Verheij HM, Rosenstein R. (1998). *Staphylococcal* lipases: molecular characterisation, secretion, and processing, *Chemistry and Physics of Lipids*, Vol. 93, 15-25.

Grbavčić, S.; Bezbradica, D.; Izrael-Živković, L.; Avramović, N.; Milosavić, N.; Karadžić, I.; Knežević-Jugović, Z. (2011). Production of lipase and protease from an indigenous *Pseudomonas aeruginosa* strain and their evaluation as detergent additives: compatibility study with detergent ingredients and washing performance. *Bioresource technology* , 2011, 102, (24), 11226-11233.

Gricajeva, A., Bendikienė, V., & Kalėdienė, L. (2016). Lipase of *Bacillus stratosphericus* L1: Cloning, expression and characterization. *International journal of biological macromolecules*, *92*, 96-104.

Gunstone F, Harwood JL, Padley FB. (1994). The lipid handbook. London: Chapman & Hall, London.

Guo, J., Sun, S., & Liu, J. (2020). Conversion of waste frying palm oil into biodiesel using free lipase A from *Candida antarctica* as a novel catalyst. *Fuel*, *267*, 117323.

Gupta N, Mehra G, Gupta R. (2004a). A glycerol-inducible thermostable lipase from *Bacillus* sp.: medium optimization by a Plackett-Burman design and by response surface methodology, *Canadian Journal of Microbiology*, Vol. 50, No. 5, 361-368.

Gupta N, Shai V, Gupta R. (2007). Alkaline lipase from a novel strain *Burkholderia multivorans*: Statistical medium optimization and production in a bioreactor, *Process Biochemistry*, Vol. 42, No. 2, 518-526.

Gupta P, Kaushal RK, Gupta R, Chimni SS and Kanwar SS. (2006). Purification and biochemical characterization of lipases of a thermophilic *Pseudomonas aeruginosa* BTS-2. *Asian Journal of Microbiology and Biotechnology*, Env Sci 8: 83-88.

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., & Chauhan, B. (2003). Microbial α-amylases: a biotechnological perspective. *Process biochemistry*, *38*(11), 1599-1616.
Gupta, R., Gupta, N and Rathi, P. (2004). Bacterial lipase: an overview of production, purification and biochemical properties. *Applied Microbiology and Biotechnology*, 64: 763-781.

Haque, E., Velmurugane, J., & Nagarajan, J. (2019). Media optimization for lipase production from *Pseudomonas otitidis* G5. *J Adv Sci Res Manag*, *4*(7).

Harwood, C. S., & Parales, R. E. (1996). The β-ketoadipate pathway and the biology of self-identity. *Annual review of microbiology*, 50(1), 553-590.

Hasan F, Shah AA, Hameed A. (2006). Industrial applications of microbial lipases, *Enzyme and Microbial Technology*, Vol. 39, No. 2, 235-251.

Hasan-Beikdashti, M., Forootanfar, H., Safiarian, M. S., Ameri, A., Ghahremani, M. H., Khoshayand, M. R., & Faramarzi, M. A. (2012). Optimization of culture conditions for production of lipase by a newly isolated bacterium *Stenotrophomonas maltophilia*. *Journal of the Taiwan Institute of Chemical Engineers*, *43*(5), 670-677.

Hassan, S. W., Abd El Latif, H. H., & Ali, S. M. (2018). Production of cold-active lipase by free and immobilized marine *Bacillus cereus* HSS: application in wastewater treatment. *Frontiers in microbiology*, *9*, 2377.

He, L., Fan, S., Müller, K., Wang, H., Che, L., Xu, S., & Bolan, N. S. (2018). Comparative analysis biochar and compost-induced degradation of di-(2-ethylhexyl) phthalate in soils. *Science of the Total Environment*, 625, 987-993.

Hegedus DD and Khachatourians GG. (1988). Production of an alkaline lipase by *Beauveria bassiana*. *Biotechnology Letters*, 10: 637-642.

Hiol A, Donzo MD, Druet D and Comeau L. (1999). Production, purification and characterization of an extracellular lipase from *Mucor hiemalis f. hiemalis*. *Enzyme and Microbial Technology*, 25: 80-87.

Hiol, A., Jonzo, M.D., Rugani, N., Druet, D., Sarda, L and Comeau, L.C. (2000). Purification and characterization of an extracellular lipase from a thermophilic *Rhizopus oryzae* strain isolated from palm fruit. *Enzyme and Microbial Technology*, 26:421-430.

Hirohara H, Mitsuda S, Ando E, Komaki R. (1985). Enzymatic preparation of optically active alcohols related to synthetic pyrethroid insecticides, Stud. Org. Chem., Vol. 22, 119-134.

Hitch, T. C., & Clavel, T. (2019). A proposed update for the classification and description of bacterial lipolytic enzymes. *PeerJ*, *7*, e7249.

Hofmeyr, S.; Meyer, C.; Warren, B. L. (2014). Serum lipase should be the laboratory test of choice for suspected acute pancreatitis. *South African Journal of Surgery*, 52, (3), 72-75.

Högberg, J., Hanberg, A., Berglund, M., Skerfving, S., Remberger, M., Calafat, A. M., & Håkansson, H. (2008). Phthalate diesters and their metabolites in human breast milk, blood or serum, and urine as biomarkers of exposure in vulnerable populations. *Environmental health perspectives*, *116*(3), 334-339.

Horikoshi, K. (1999). Alkaliphiles: some applications of their products for biotechnology. *Microbiology and molecular biology reviews*, 63(4), 735-750.

Hou, C. T., & Johnston, T. M. (1992). Screening of lipase activities with cultures from the agricultural research service culture collection. *Journal of the American Oil Chemists' Society*, 69(11), 1088-1097.

Houde, A., Kademi, A., & Leblanc, D. (2004). Lipases and their industrial applications. *Applied biochemistry and biotechnology*, *118*(1), 155-170.

Huang, H., Zhang, X. Y., Chen, T. L., Zhao, Y. L., Xu, D. S., & Bai, Y. P. (2019). Biodegradation of structurally diverse phthalate esters by a newly identified esterase with catalytic activity toward di (2-ethylhexyl) phthalate. *Journal of agricultural and food chemistry*, 67(31), 8548-8558.

Ilesanmi, O. I., Adekunle, A. E., Omolaiye, J. A., Olorode, E. M., & Ogunkanmi, A. L. (2020). Isolation, optimization and molecular characterization of lipase producing bacteria from contaminated soil. *Scientific African*, *8*, e00279.

Immanuel, G., Esakkiraj, P., Jebadhas, A., Iyapparaj, P., & Palavesam, A. (2008). Investigation of lipase production by milk isolate *Serratia rubidaea*. *Food Technology and Biotechnology*, 46(1), 60-65.

Iqbal, S. A., & Rehman, A. (2015). Characterization of lipase from *Bacillus subtilis* I-4 and its potential use in oil contaminated wastewater. *Brazilian archives of biology and technology*, *58*, 789-797.

Ishiguro, N., & Sato, G. (1979). The distribution of plasmids determining citrate utilization in citrate-positive variants of *Escherichia coli* from humans, domestic animals, feral birds and environments. *Epidemiology & Infection*, *83*(2), 331-344.

Ito, T., Kikuta, H., Nagamori, E., Honda, H., Ogino, H., Ishikawa, H., & Kobayashi, T. (2001). Lipase production in two-step fed-batch culture of organic solvent-tolerant Pseudomonas aeruginosa LST-03. *Journal of bioscience and bioengineering*, 91(3), 245-250.

Iverson, W. G., & Millis, N. F. (1974). A method for the detection of starch hydrolysis by bacteria. *Journal of Applied Bacteriology*, *37*(3), 443-446.

Jaeger, K. E., Dijkstra, B. W., & Reetz, M. T. (1999). Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annual Reviews in Microbiology*, 53(1), 315-351.

Jaeger, K., Reetz, T. (1998). Microbial lipases from versatile tools for biotechnology. TIBTECH. 16, 396–403.

Jaeger, K.-E.; Eggert, T. (2002). Lipases for biotechnology. *Current opinion in Biotechnology*, 13, (4), 390-397.

Janssen, P.H., Monk, C.R and Morgan, H.W. (1994). A thermophilic, lipolytic *Bacillus* sp. and continuous assay of its p-nitrophenyl-palmitate esterase activity. *FEMS Microbiology Letters*, 120:195-200.

Javed, Saira, Farrukh Azeem, Sabir Hussain, Ijaz Rasul, Muhammad Hussnain Siddique, Muhammad Riaz, Muhammad Afzal, Ambreen Kouser, and Habibullah Nadeem (2018). Bacterial lipases: a review on purification and characterization. *Progress in biophysics and molecular biology*, *132*, 23-34.

Jayaraman, G. C., Penders, J. E., & Burne, R. A. (1997). Transcriptional analysis of the *Streptococcus mutans* hrcA, grpE and dnaK genes and regulation of expression in response to heat shock and environmental acidification. *Molecular microbiology*, 25(2), 329-341.

Jinwal, U. K., Roy, U., Chowdhury, A. R., Bhaduri, A. P., & Roy, P. K. (2003). Purification and characterization of an alkaline lipase from a newly isolated *Pseudomonas mendocina* PK-12CS and chemoselective hydrolysis of fatty acid ester. *Bioorganic & medicinal chemistry*, 11(6), 1041-1046.

Jones, A., & Richards, T. (1952, April). Night blue and Victoria blue as indicators in lipolysis media. In Proceedings of the Society for Applied Bacteriology (Vol. 15, No. 1, pp. 82-93). Oxford, UK: Blackwell Publishing Ltd.

Jooyandeh, H.; Kaur, A.; Minhas, K. (2009). Lipases in dairy industry: a review. *Journal of Food Science and Technology*, 46, (3), 181-189.

Joseph, B., Ramteke, P. W., Thomas, G., & Shrivastava, N. (2007). Cold-active microbial lipases: a versatile tool for industrial applications. *Biotechnology and Molecular Biology Reviews*, *2*(2), 39-48.

Jozo T. (1988). Production of optically active β-monoalkyl malate and optically active isoserine, Japanese patent JPJ63137687.

Jurado E, Bravo V, Luzon G, Serrano MF, Roman MG, Vaz DA, Vicaria JM. (2007). Hard-surface cleaning using lipases: enzyme–surfactant interactions and washing tests, *Journal of Surfactants and Detergents*, Vol. 10,61-70.

Kademi A, Danielle L, Ajain H. (2005). Lipases, *Enzyme Technology*. India: Asiatech Publishers. 297-318.

Kai, W., & Peisheng, Y. (2016). Optimization of lipase production from a novel strain *Thalassospira permensis* M35-15 using response surface methodology. *Bioengineered*, *7*(5), 298-303.

Kakde, R. B., & Chavan, A. M. (2011). Extracellular lipase enzyme production by seed-borne fungi under the influence of physical factors. *International Journal of Biology*, *3*(1), 94.

Kakugawa, K., Shobayashi, M., Suzuki, O., & Miyakawa, T. (2002). Purification and characterization of a lipase from the glycolipid-producing yeast *Kurtzmanomyces* sp. I-11. *Bioscience, biotechnology, and biochemistry*, *66*(5), 978-985.

Kambourova, M., Kirilova, N., Mandeva, R. and Derekova, A. (2003). Purification and properties of thermostable lipase from a thermophilic *Bacillus stearothermophilus* MC 7. *Journal of Molecular Catalysis B: Enzymatic*, *22*(5-6), 307-313.

Kanimozhi, K., Devairrakam, E. W. J., & Jegadeesh kumar, D. (2013). Decolorization of Leather effluent by lipase producing *Bacillus* sp. *J. Acad. Indus. Res*, *1*(12), 813.

Kanjanavas, P.; Khuchareontaworn, S.; Khawsak, P.; Pakpitcharoen, A.; Pothivejkul, K.; Santiwatanakul, S.; Matsui, K.; Kajiwara, T.; Chansiri, K. (2010). Purification and characterization of organic solvent and detergent tolerant lipase from thermotolerant *Bacillus* sp. RN2. *International journal of molecular sciences*, 11, (10), 3783-3792.

Kanwar, L and Goswami, P. (2002). Isolation of *Pseudomonas* lipase produced in pure hydrocarbon substrate and its application in the synthesis of isoamyl acetate using membrane immobilized lipase. *Enzyme and Microbial Technology*, 31: 727–735.

Kar M, Ray L, Chattopadhyay P. (1996). Isolation and identification of alkaline thermostable lipase producing microorganism and some properties of crude enzyme, *Indian journal of experimental biology*, Vol. 34, 535-538.

Karadzic, I., Masui, A., Zivkovic, L. I., & Fujiwara, N. (2006). Purification and characterization of an alkaline lipase from *Pseudomonas aeruginosa* isolated from putrid mineral cutting oil as component of metalworking fluid. *Journal of Bioscience and Bioengineering*, 102(2), 82-89.

Karan RKS, Sinha R, Khare SK. (2012). Halophilic microorganisms as sources of novel enzymes. Microorganisms in sustainable agriculture and biotechnology. TJ Satyanarayana, Bhavdish Narain; Anil Prakash, Springer Netherlands: 555–579.

Kasana, R.C., Kaur, B and Yadav, S.K. (2008). Isolation and identification of a psychrotrophic *Acinetobacter* sp. CR9 and characterization of its alkaline lipase. *Journal of Basic Microbiology*, 48: 207-212.

Kavitha, M., & Shanthi, C. (2017). Alkaline thermostable cold active lipase from halotolerant *Pseudomonas* sp. VITCLP4 as detergent additive.

Kazlauskas RJ, Bornscheuer UT. (1998). Biotransformations with lipases. In: Rehm HJ, Pihler G, Stadler A, Kelly PJW, editors. Biotechnology. vol. 8. New York: VCH, 37–192.

Khemika L, Angkhameen B, Hataichanoke N. (2012). Investigation of Isolated Lipase Producing Bacteria from Oil-contaminated Soil with Proteomic Analysis of its Proteins Responsive to Lipase Inducer. *Journal of Biological Sciences* , 12: 161-167.

Khosla, K., Rathour, R., Maurya, R., Maheshwari, N., Gnansounou, E., Larroche, C., & Thakur, I. S. (2017). Biodiesel production from lipid of carbon dioxide sequestrating bacterium and lipase of psychrotolerant *Pseudomonas* sp. ISTPL3 immobilized on biochar. *Bioresource technology*, *245*, 743-750.

Kim, H. K., Sung, M. H., Kim, H. M., & Oh, T. K. (1994). Occurrence of thermostable lipase in thermophilic *Bacillus* sp. strain 398. *Bioscience, biotechnology, and biochemistry*, 58(5), 961-962.

Kim, H.K., Lee, J.K., Kim, H and Oh, T.K. (1996). Characterization of an alkaline lipase from *Proteus vulgaris* K80 and the DNA sequence of the encoding gene. *FEMS Microbiology Letters*, 135:117–21

Kim, H.K., Park, S.Y., Lee, J.K. and Oh, T.K. (1998). Gene cloning and characterization of thermostable lipase from *Bacillus stearothermophilus* L1. *Bioscience, biotechnology, and biochemistry*, 62(1), 66-71.

Kim, J.-H.; Bhatia, S. K.; Yoo, D.; Seo, H. M.; Yi, D.-H.; Kim, H. J.; Lee, J. H.; Choi, K.-Y.; Kim, K. J.; Lee, Y. K. (2015). Lipase-Catalyzed Production of 6-O-cinnamoylsorbitol from D-sorbitol and Cinnamic Acid Esters. *Applied biochemistry and biotechnology*, 176, (1), 244-252.

Kim, M., Kim, H., Lee, J and Oh, T. (2000). Thermostable lipase of *Bacillus stearothermophilus:* high level production, purification and calcium dependent thermostability. *Bioscience, biotechnology, and biochemistry*, 64 (2): 280-286.

Kim, S. H.; Kim, S.-j.; Park, S.; Kim, H. K. (2013). Biodiesel production using crosslinked *Staphylococcus haemolyticus* lipase immobilized on solid polymeric carriers. *Journal of Molecular Catalysis B: Enzymatic*, 2013, 85, 10-16.

Kiran GS, Shanmughapriya S, Jayalakshmi J, Selvin J, Gandhimathi R, Sivaramakrishnan S, Arunkumar M, Thangavelu T, and Natarajaseenivasan K.(2008). Optimization of extracellular psychrophilic alkaline lipase produced by marine *Pseudomonas* sp. (MSI057), *Bioprocess and Biosystem Engineering*, Vol. 31, 483-492.

Ko, H. G., Park, S. H., Kim, S. H., Park, H. G., & Park, W. M. (2005). Detection and recovery of hydrolytic enzymes from spent compost of four mushroom species. *Folia microbiologica*, *50*(2), 103.

Kojima Y, Yokoe M and Mase T. (1994). Purification and characterization of an alkaline lipase from *Pseudomonas fluorescens* AK 102. *Bioscience, biotechnology, and biochemistry*,1564-1568

Kojima, Y., & Shimizu, S. (2003). Purification and characterization of the lipase from *Pseudomonas fluorescens* HU380. *Journal of bioscience and bioengineering*, *96*(3), 219-226.

Kok, R.G., Thor, J.J.V., Roodzant, I.M.N., Brouwer, M.B.W., Egmond, M.R., Nudel, C.B., Vosman, B and Hellingwer, K. J. (1995).Characterization of the extracellular lipase lipA of *Acinetobacter colcoaceticus* BD413 and sequence analysis of the cloned structural gene. *Molecular Microbiology,* 15:803-18.

Kolmos, H. J., & Schmidt, J. (1987). Failure to detect hydrogen-sulphide production in lactose/sucrose-fermenting *Enterobacteriaceae*, using triple sugar iron agar. *Acta Pathologica Microbiologica Scandinavica Series B: Microbiology*, *95*(1-6), 85-87.

Kristjansson, M. M., & Kinsella, J. E. (1991). Protein and enzyme stability: structural, thermodynamic, and experimental aspects. In *Advances in food and nutrition research* (Vol. 35, 237-316). Academic Press.

Ktata, A., Karray, A., Mnif, I., & Bezzine, S. (2020). Enhancement of *Aeribacillus pallidus* strain VP3 lipase catalytic activity through optimization of medium composition using Box-Behnken design and its application in detergent formulations. *Environmental Science and Pollution Research*, *27*(11), 12755-12766.

Kulkarni N, Gadre RV. (2002). Production and Properties of an Alkaline, Hermophilic Lipase from *Pseudomonas fluorescens* NS2W. *Journal of Industrial Microbiology and Biotechnology*, 28: 344-348.

Kumar, A., Mukhia, S., Kumar, N., Acharya, V., Kumar, S., & Kumar, R. (2020). A broad temperature active lipase purified from a psychrotrophic bacterium of sikkim himalaya with potential application in detergent formulation. *Frontiers in bioengineering and biotechnology*, *8*, 642.

Kumar, D., Kumar, L., Nagar, S., Raina, C., Parshad, R., & Gupta, V. K. (2012). Screening, isolation and production of lipase/esterase producing *Bacillus* sp. strain DVL2 and its potential evaluation in esterification and resolution reactions. *Archives of Applied Science Research*, *4*(4), 1763-1770.

Kumar, K., Usha, K.Y., Satyanarayana, S.V., Sailaja, V. (2014). Characterization of partially purified lipase from *Saccharomyces cerevisiae*. *Int. J. Pharm. Pharm. Sci*. 6, 514-517.

Kumar, R., Sharma, A., Kumar, A., & Singh, D. (2012). Lipase from *Bacillus pumilus* RK31: Production, purification and some properties. *World Appl Sci J*, *16*(7), 940-948.

Kumari, A. and Gupta, R. (2012). Extracellular expression and characterization of thermostable lipases, LIP8, LIP14 and LIP18, from *Yarrowia lipolytica*. *Biotechnology letters*, *34*(9), 1733-1739.

Kuppamuthu, K., Soundiraraj, S., & Palanisamy, K. (2021). Utilization of whey as a cheap substrate for the optimization of lipase production by *Bacillus subtilis* B10 isolated from dairy industry. *Journal of Microbiology, Biotechnology and Food Sciences, 2021*, 193-198.

Laachari, F.; El Bergadi, F.; Sayari, A.; Elabed, S.; Mohammed, I.; Harchali, E. H.; Ibnsouda, S. K. (2015). Biochemical characterization of a new thermostable lipase from *Bacillus pumilus* strain. *Turkish Journal of Biochemistry*, 40, (1), 8-14.

Larsen H. (1986). Halophilic and halotolerant microorganisms-an overview and historical perspective. *FEMS Microbiology Reviews*, 39: 3–7.

Larsen, M. D., Kristiansen, K. R., and Hansen, T. K. (1998). Characterization of the proteolytic activity of starter cultures of *Penicillium roqueforti* for production of blue veined cheeses. *International Journal of Food Microbiology*, 43 (3):215-221

Lee, D., Kok, Y., Kim, K., Kim, B., Choi, H., Kim, D., Suhartono, M.T and Pyun, Y. (1999). Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. *FEMS Microbiology Letters*, 179:393- 400.

Lee, L. P., Karbul, H. M., Citartan, M., Gopinath, S. C., Lakshmipriya, T., & Tang, T. H. (2015). Lipase-secreting *Bacillus* species in an oil-contaminated habitat: promising strains to alleviate oil pollution. *BioMed research international*, *2015*.

Lee, S. Y., & Rhee, J. S. (1993). Production and partial purification of a lipase from *Pseudomonas putida* 3SK. *Enzyme and Microbial Technology*, 15(7), 617-623.

Lee, W.M., Kim, K.J., Kim, M.G and Lee, S.B. (1995). Enzymatic resolution of racemic ibuprofen esters: effects of organic cosolvents and temperature. *Journal of Fermentation and Bioengineering*, 6:613- 615.

Lentzen G, Schwarz T. (2006). Extremolytes: natural compounds from extremophiles for versatile applications. *Applied Microbiology and Biotechnology*, 72(4):623–634.

LESUISSE, E., SCHANCK, K., & COLSON, C. (1993). Purification and preliminary characterization of the extracellular lipase of Bacillus subtilis 168, an extremely basic pH-tolerant enzyme. *European Journal of Biochemistry*, 216(1), 155-160.

Li, C. Y., Chen, S. J., Cheng, C. Y., & Chen, T. L. (2005). Production of Acinetobacter radioresistens lipase with repeated fed-batch culture. *Biochemical Engineering Journal*, 25(3), 195-199.

Li X, Yu H-Y. (2014). Characterization of an organic solvent-tolerant lipase from *Haloarcula* sp. G41 and its application for biodiesel production. *Folia Microbiologica*.

Li, C.Y., Cheng, C.Y., Chen, T.L. (2001). Production of *Acinetobacter radioresistens* lipase using Tween 80 as the carbon source. *Enzyme and Microbial Technology*, 29, 258–263.

Li, H. and Zhang, X. (2005). Characterization of thermostable lipase from thermophilic *Geobacillus* sp. TW1. *Protein Expression and Purification*, 42(1), 153-159.

Li, J., Shen, W., Fan, G., & Li, X. (2018). Screening, purification and characterization of lipase from *Burkholderia pyrrocinia* B1213. *3 Biotech*, *8*(9), 1-12.

Li, J., Zhang, J., Yadav, M. P., & Li, X. (2019). Biodegradability and biodegradation pathway of di-(2-ethylhexyl) phthalate by *Burkholderia pyrrocinia* B1213. *Chemosphere*, *225*, 443-450.

Li, X., & Yu, H. Y. (2012). Characterization of a novel extracellular lipase from a halophilic isolate, *Chromohalobacter* sp. LY7-8. *African Journal of Microbiology Research*, 6(14), 3516-3522.

Li, X., & Yu, H. Y. (2013). Characterization of a halostable endoglucanase with organic solvent-tolerant property from *Haloarcula* sp. G10. *International journal of biological macromolecules*, *62*, 101-106.

Li, X., Qian, P., Wu, S. G., & Yu, H. Y. (2014). Characterization of an organic solvent-tolerant lipase from *Idiomarina* sp. W33 and its application for biodiesel production using Jatropha oil. *Extremophiles*, *18*(1), 171-178.

Li, Y., Zhang, Q., Fang, J., Ma, N., Geng, X., Xu, M. & Jia, X. (2020). Hepatotoxicity study of combined exposure of DEHP and ethanol: A comprehensive analysis of transcriptomics and metabolomics. *Food and Chemical Toxicology*, *141*, 111370.

Liang, D. W., Zhang, T., Fang, H. H., & He, J. (2008). Phthalates biodegradation in the environment. *Applied microbiology and Biotechnology*, *80*(2), 183-198.

Liebeton K, Zacharias A, Jaeger KE. (2001). Disulfide bond in *Pseudomonas aeruginosa* lipase stabilizes the structure but is not required for interaction with its foldase, *Journal of Bacteriology*, Vol. 183, No. 2, 597-603.

Lima, R. T., Alves, A. M., de Paula, A. V., de Castro, H. F., & Andrade, G. S. (2019). Mycelium-bound lipase from *Penicillium citrinum* as biocatalyst for the hydrolysis of vegetable oils. *Biocatalysis and Agricultural Biotechnology*, *22*, 101410.

Lima, V.M.G., Krieger, N., Sarquis, M.I.M., Mitchell, D.A., Ramos, L.P and Fontana, J.D. (2003). Effect of nitrogen and carbon sources on lipase production by *Penicillium aurantiogriseum. Food Technology and Biotechnology*, 41(2):105-110.

Lin SF, Chiou C M and Tsai Y C. (1995). Effect of Triton X-100 on alkaline lipase production by *Pseudomonas pseudoalkaligenes* F-111. *Biotechnology Letters*, 17: 959-962.

Lin, E.S., Wang, C.C and Sung, S.C. (2006). Cultivating conditions influence lipase production by the edible Basidiomycete *Antrodia cinnamomea* in submerged culture. *Enzyme and Microbial Technology*, 39: 98-102.

Lin, S. F., Chiou, C. M., Yeh, C. M., & Tsai, Y. C. (1996). Purification and partial characterization of an alkaline lipase from *Pseudomonas pseudoalcaligenes* F-111. *Applied and Environmental Microbiology*, 62(3), 1093-1095.

Liu IL, Tsai SW. (2003). Improvements in lipase production and recovery form *Acinetobacter radioresistens* in presence of polypropylene powders filled with carbon sources, *Applied biochemistry and biotechnology*, Vol. 104, 129-140.

Lomthaison, K., Buranarom, A and Niamsup, H. (2012). Investigation of isolated lipase producing bacteria from oil contaminated soil with proteomic analysis of its protein responsive to lipase inducer, *Journal of Biological Sciences,* 12(3): 161-167.

Lonetto MA, Brown KL, Rudd KE, Buttner MJ. (1994). Analysis of the Streptomyces coelicolor sigE gene reveals the existence of a subfamily of eubacterial RNA polymerase σ factor involved in the regulation of extracytoplasmic functions. Proc Natl Acad Sci USA; 91: 618 7573–7577

Lopes M, Leitao AL, Regalla M, Marques JJ, Carrondo MJ, Crespo MT. (2002). Characterization of a highly thermostable extracellular lipase from *Lactobacillus plantarum*, *International Journal of Food Microbiology*, Vol. 76, 107-115.

Lotrakul, P and Dharmsthiti, S. (1997). Lipase pryoooduction by *Aeromonas sobria* LP004 in a medium containing whey and soybean meal. *World Journal of Microbiology and Biotechnology*, 13: 163-166.

Lowry, R.R. and Tinsley, I.J. (1976). Rapid colorimetric determination of free fatty acids. *Journal of American Oil chemistry*. Soc.53, 470-472

Lü, H., Mo, C. H., Zhao, H. M., Xiang, L., Katsoyiannis, A., Li, Y. W., & Wong, M. H. (2018). Soil contamination and sources of phthalates and its health risk in China: a review. *Environmental research*, *164*, 417-429.

Madern, D., Ebel, C., & Zaccai, G. (2000). Halophilic adaptation of enzymes. *Extremophiles*, *4*(2), 91-98.

Mahadik, N. D., Puntambekar, U. S., Bastawde, K. B., Khire, J. M and Gokhale, D. V. (2002). Production of acidic lipase by *Aspergillus niger* in solid state fermentation, *Process Biochemistry*, 38(5): 715-721.

Mahanta N, Gupta A, Khare SK. (2008). Production of protease and lipase by solvent tolerant *Pseudomonas aeruginosa* PseA in solid-state fermentation using Jatropha curcas seed cake as substrate, *Bioresource Technology*, Vol. 99, 1729-1735.

Makhzoum M, Knapp JS and Dwusu RK. (1995). Factors affecting growth and extracellular lipase production by *Pseudomonas fluorescence* 2D. *Food Microbiology*, 12: 277-290.

Makhzoum, A., Owusu-Apenten, R.K and Knapp, J.S. (1996). Purification and properties of lipase from *Pseudomonas fluorescens* strain 2D. *Int. Diary J*., 6: 459-472.

Malekabadi, S., Badoei-Dalfard, A., & Karami, Z. (2018). Biochemical characterization of a novel cold-active, halophilic and organic solvent-tolerant lipase from *B. licheniformis* KM12 with potential application for biodiesel production. *International journal of biological macromolecules*, *109*, 389-398.

Manan, A., Bazai, Z. A., Fan, J., Yu, H., & Li, L. (2018). The Nif3-family protein YqfO03 from *Pseudomonas syringae* MB03 has multiple nematicidal activities against Caenorhabditis elegans and Meloidogyne incognita. *International journal of molecular sciences*, 19(12), 3915.

Margesin R, Feller G, Gerday C, Rusell N. (2002). Cold adapted Microorganisms: Adaptation strategies and Biotechnological Potential. In: Bitton (ed). The Encyclopedia of Environmental Microbiology, John Wiley & Sons, New York, 871-885

Margesin, R., Neuner, G., & Storey, K. B. (2007). Cold-loving microbes, plants, and animals—fundamental and applied aspects. *Naturwissenschaften*, *94*(2), 77-99.

Martinelle, M., Holmquist, M., & Hult, K. (1995). On the interfacial activation of *Candida antarctica* lipase A and B as compared with *Humicola lanuginosa* lipase. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 1258(3), 272-276.

Martinelle M, Hult K. (1995). Kinetics of acyl transfer reactions in organic media catalyzed by *Candida antarctica* lipase B, *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*. Vol. 1251, 191-7

Massadeh, Muhannad I., and Fatima M. Sabra. (2011). Production and characterization of lipase from *Bacillus stearothermophilus*." *African Journal of Biotechnology* 10.61 : 13139-13146.

Masse, L., Kennedy, K. J., & Chou, S. P. (2001). The effect of an enzymatic pretreatment on the hydrolysis and size reduction of fat particles in slaughterhouse wastewater. Journal of Chemical Technology & Biotechnology: *International Research in Process, Environmental & Clean Technology*, 76(6), 629-635.

Mayordomo, I., Randez-Gil, F., & Prieto, J. A. (2000). Isolation, purification, and characterization of a cold-active lipase from *Aspergillus nidulans*. *Journal of Agricultural and Food Chemistry*, 48(1), 105-109.

Mazhar, H., Abbas, N., Ali, S., Sohail, A., Hussain, Z., & Ali, S. S. (2017). Optimized production of lipase from *Bacillus subtilis* PCSIRNL-39. *African Journal of Biotechnology*, *16*(19), 1106-1115.

McKellar, R. C. (1986). A rapid colorimetric assay for the extracellular lipase of *Pseudomonas fluorescens* B52 using β-naphthyl caprylate. *Journal of dairy research*, *53*(1), 117-127.

McPherson, A., Larson, S. B., & Kalasky, A. (2020). The Crystal Structures of *Thermomyces (Humicola) Lanuginosa* Lipase in Complex with Enzymatic Reactants. *Current Enzyme Inhibition*, *16*(3), 199-213.

Mehta, A., Kumar, R., & Gupta, R. (2012). Isolation of lipase producing thermophilic bacteria: Optimization of production and reaction conditions for lipase from *Geobacillus* sp. Acta microbiologica et immunologica Hungarica, *59*(4), 435-450.

Meng, X.Z., Wang, Y., Xiang, N., Chen, L., Liu, Z., Wu, B., Dai, X., Zhang, Y.H., Xie, Z., Ebinghaus, R. (2014). Flow of sewage sludge-borne phthalate esters (PAEs) from human release to human intake: implication for risk assessment of sludge applied to soil. Science of Total Environment, 476-477, 242–249

Messias, J.M., da Costa, B.Z., de Lima, V.M., Dekker, R.F., Rezende, M.I., Krieger, N., Barbosa, A.M. (2009). Screening *Botryosphaeria* species for lipases: production of lipasbhushane by *Botryosphaeria ribis* EC-01 grown on soybean oil and other carbon sources. *Enzyme and Microbial Technology*, 45, 426–431.

Mhetras NC, Bastawde KB, Gokhale DV. (2009). Purification and characterization of acidic lipase from *Aspergillus niger* NCIM 1207. *Bioresource Technology*, 100: 1486–1490

Mobarak-Qamsari, E., Kasra-Kermanshahi, R and Moosavi-nejad, Z. (2011). Isolation and identification of a novel, lipase producing bacterium, *Pseudomnas aeruginosa* KM110, *Iranian Journal of Microbiology*, 3(2): 92 -98.

Moh'd A, S., & Wiegel, J. (2010). Effects of detergents on activity, thermostability and aggregation of two alkalithermophilic lipases from *Thermosyntropha lipolytica*. *The open biochemistry journal*, *4*, 22.

Mohamed, S. S., Ahmed, H. M., El-Bendary, M. A., Moharam, M. E., & Amin, H. A. (2021). Response surface methodology for optimization of *Rhizopus stolonifer* 1aNRC11 mutant F whole-cell lipase production as a biocatalyst for methanolysis of waste frying oil. *Biocatalysis and Biotransformation*, *39*(3), 232-240.

Molinari F, Brenna O, Valenti M and Aragozzini F. (1996). Isolation of a novel carboxyesterase from *Bacillus coagulans* with high enantioselectivity towards racemic esters of 1, 2, 0-isopropylindene glycerol. *Enzyme and Microbial Technology*,19: 555-556.

Monteiro, R. R., Virgen-Ortiz, J. J., Berenguer-Murcia, Á., da Rocha, T. N., dos Santos, J. C., Alcántara, A. R., & Fernandez-Lafuente, R. (2021). Biotechnological relevance of the lipase A from *Candida antarctica*. *Catalysis Today*, *362*, 141-154.

Montero, L., Ibáñez, E., Russo, M., Rastrelli, L., Cifuentes, A., & Herrero, M. (2017). Focusing and non-focusing modulation strategies for the improvement of on-line two-dimensional hydrophilic interaction chromatography× reversed phase profiling of complex food samples. *Analytica chimica acta*, *985*, 202-212.

Morita, R. Y. (1975). Psychrophilic bacteria. *Bacteriological reviews*, *39*(2), 144.

Mosbah H, Sayari A, Mejdoub H, Dhouib H, Gargouri Y. (2005). Biochemical and molecular characterization of *Staphylococcus xylosus* lipase, *Biochimica et Biophysica Acta (BBA)*-General Subjects, Vol. 25. 282-91.

MU, N., Mehar, J. G., Mudliar, S. N., & Shekh, A. Y. (2019). Recent advances in microalgal bioactives for food, feed, and healthcare products: commercial potential, market

space, and sustainability. *Comprehensive reviews in food science and food safety*, *18*(6), 1882-1897.

Muderhwa JM, Ratomahenina R, Pina M, Graille M and Galzy P. (1986). Purification and properties of lipase from *Rhodotorula pilmanae* Hedrik and Bruke. *Applied Microbiology and Biotechnology*, 23: 348-354.

Muralidhar RV, Chirumamilla RR, Marchant R, Ramachandran VN, Ward OP, Nigam P. (2002). Understanding lipase stereoselectivity, *World Journal of Microbiology and Biotechnology*, Vol. 18, No. 2, 81-97.

Mussatto, S.I., Ballesteros, L.F., Martins, S., Teixeira, J.A. (2012). Use of agro-industrial wastes in solid-state fermentation processes. Industrial waste, 274.

Nadeem, H., H Rashid, M., & H Siddique, M. (2015). Effect of Mg2+ and Al3+ ions on thermodynamic and physiochemical properties of *Aspergillus niger* Invertases. *Protein and peptide letters*, *22*(8), 743-749.

Nadeem, H., Rashid, M. H., Riaz, M., Asma, B., Javed, M. R., & Perveen, R. (2009). Invertase from hyper producer strain of *Aspergillus niger*: physiochemical properties, thermodynamics and active site residues heat of ionization. *Protein and Peptide Letters*, *16*(9), 1098-1105.

Nahurira, R., Ren, L., Song, J., Jia, Y., Wang, J., Fan, S., & Yan, Y. (2017). Degradation of di (2-ethylhexyl) phthalate by a novel *Gordonia alkanivorans* strain YC-RL2. *Current microbiology*, *74*(3), 309-319.

Nakajima M, Snape J, Khare SK. (2000). In: Gupta MN, editor. Method in non-aqueous enzymology. Basel: Birkhauser Verlag; 52– 69.

Nasaruddin RR, Alam MZ, Jami MS. (2014). Evaluation of solvent system for the enzymatic synthesis of ethanol-based biodiesel from sludge palm oil (SPO). *Bioresource Technology*; 154: 155–161.

Nathan, V. K., & Rani, M. E. (2020). A cleaner process of deinking waste paper pulp using *Pseudomonas mendocina* ED9 lipase supplemented enzyme cocktail. *Environmental Science and Pollution Research*, *27*(29), 36498-36509.

Nawani N, Kaur J. (2000). Purification, characterization and thermostability of a lipase from a thermophilic *Bacillus* sp. J33, *Molecular and cellular Biochemistry*, Vol. 206, 91-96.

Norouzian, D., Akbarzadeh, A., Scharer, J. M., & Young, M. M. (2006). Fungal glucoamylases. *Biotechnology advances*, *24*(1), 80-85.

Nthangeni MB, Patterton HG, Tonder AV, Vergeer WP, Litthauer D. (2001). Over expression and properties of a purified recombinant *Bacillus licheniformis* lipase: a comparative report on Bacillus lipases, *Enzyme and Microbial Technology*, Vol. 28, 705-712.

Oh B, Kim H, Lee J, Kang S, Oh T. (1999). *Staphylococcus haemolyticus* lipase: biochemical properties, substrate specificity and gene cloning, *FEMS Microbiology Letters*, Vol. 179, 385-92.

Ohnishi, K., Yoshida, Y., Toita, J and Sekiguchi, J. (1994b). Purification and characterization of a novel lipolytic enzyme from *Aspergillus oryzae*. *Journal of Fermentation and Bioengineering*, 78: 413–419.

Okamura-Abe, Y., Abe, T., Nishimura, K., Kawata, Y., Sato-Izawa, K., Otsuka, Y. (2016). Beta-ketoadipic acid and muconolactone production from a lignin-related aromatic compound through the protocatechuate 3,4-metabolic pathway. *Journal of Bioscience and Bioengineering*, 121 (6), 652e658.

Okumura S, Iwai M, Tsujisaka Y. (1979). Synthesis of various kinds of esters by four microbial lipases. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 575:156–65.

Oliveira, F., Moreira, C., Salgado, J.M., Abrunhosa, L., Venâncio, A., Belo, I., (2016). Olive pomace valorization by *Aspergillus* species: lipase production using solid-state fermentation. *Journal of Science Food and Agriculture,* 96 (10), 3583-3589.

Olukoshi ER, Packter NM. (1994) Importance of stored triacylglycerols in Streptomyces — possible carbon source far antibiotics. Microbiology-UK 140:931–943

Olusesan, A. T., Azura, L. K., Forghani, B., Bakar, F. A., Mohamed, A. K. S., Radu, S.,& Saari, N. (2011). Purification, characterization and thermal inactivation kinetics of a non-regioselective thermostable lipase from a genotypically identified extremophilic *Bacillus subtilis* NS 8. *New biotechnology*, 28(6), 738-745.

Omar IC, Hayashi M and Nagai S. (1987). Purification and some properties of a thermostable lipase from *Humicola lanuginosa* No. 3. *Agricultural and biological chemistry*, 51: 37-45.

Ooijkaas, L.P., Weber, F.J., Buitelaar, R.M., Tramper J and Rinzema A. (2000). Defined media and inert supports: their potential as solid-state fermentation production systems. *Trends in Biotechnology*, 18:356 -360.

Oren A. (2002). Adaptation of halophilic archaea to life at high salt concentrations. Salinity: environments–plants–molecules. AL Lauchli, U. Dordrecht, Kluwer Academic: 81–96.

Ortega, N., De Diego, S., Perez-Mateos, M., & Busto, M. D. (2004). Kinetic properties and thermal behaviour of polygalacturonase used in fruit juice clarification. *Food chemistry*, 88(2), 209-217.

Oves, M., Qari, H. A., Felemban, N. M., Khan, M. Z., Rehan, Z. A., & Ismail, I. M. (2017). *Marinobacter lipolyticus* from Red Sea for lipase production and modulation of silver nanomaterials for anti-candidal activities. *IET nanobiotechnology*, *11*(4), 403-410.

Owusu, R. K., Makhzoum, A., & Knapp, J. S. (1992). Heat inactivation of lipase from psychrotrophic *Pseudomonas fluorescens* P38: activation parameters and enzyme stability at low or ultra-high temperatures. *Food chemistry*, *44*(4), 261-268.

Ozcan B, Ozyilmaz G, Cokmus C, Caliskan M. (2009). Characterization of extracellular esterase and lipase activities from five halophilic archaeal strains. *Journal of Industrial Microbiology and biotechnology*; 36:105–110.

Pabai, F., Kermasha, S and Morin, A. (1995). Interestrification of butter fat by partily purified extracellular lipase from *Pseudomonas putida*, *Aspergillus niger* and *Rhyzopus oryza*, *World Journal of Microbiology and Biotechnology*, 11: 669 - 677.

Pace, C. N. (1975). The stability of globular proteins. *CRC Critical Reviews in Biochemistry*, 3, 1–43.

Palekar, A.A., Vasudevan, P.T. and Yan, S (2000). Purification of lipase: A review, Biocatalysis and Biotransformation. 18: 177-200.

Pan, D.; Pham, C. T. N.; Weilbaecher, K. N.; Tomasson, M. H.; Wickline, S. A.; Lanza, G. M. (2016). Contact-facilitated drug delivery with Sn2 lipase labile prodrugs optimize targeted lipid nanoparticle drug delivery. Wiley Interdisciplinary Reviews-Nanomedicine and Nanobiotechnology, 8, (1), 85-106.

Pandey, A., Benjamin, S., Soccol, C. R., Nigam, P., Krieger, N., & Soccol, V. T. (1999). The realm of microbial lipases in biotechnology. *Biotechnology and applied biochemistry*, *29*(2), 119-131.

Papamichael, E. M., Stergiou, P. Y., Foukis, A., Kokkinou, M., & Theodorou, L. G. (2012). Effective kinetic methods and tools in investigating the mechanism of action of specific hydrolases. Medicinal chemistry and drug design. Rijeka: INTECH Open Science, 235-74.

Papaparaskevas, D., Christakopoulos, P., Kekos, D., & Macris, B. J. (1992). Optimizing production of extracellular lipase from *Rhodotorula glutinis*. *Biotechnology Letters*, *14*(5), 397-402.

Patel, S. K., Pratap, C. B., Verma, A. K., Jain, A. K., Dixit, V. K., & Nath, G. (2013). *Pseudomonas fluorescens*-like bacteria from the stomach: a microbiological and molecular study. *World Journal of Gastroenterology: WJG*, *19*(7), 1056.

Pathak, A. P., Kamble, G. T., Jadhav, S. R., & Sarsar, M. S. (2015). Isolation and biochemical Characterization of potential thermostable Lipase producer from industrial effluent of oil, dairy and paper industry. *Int. J. Adv. Pharm. Biol. Chem*, *4*(4), 825-828.

Patil, K. J.; Chopda, M. Z.; Mahajan, R. T. (2011). Lipase biodiversity. *Indian Journal of Science and Technology*, 4, (8), 971-982.

Pérez D, Martín S, Fernández-Lorente G, Filice M, Guisán JM, Ventosa A, García MT, Mellado E. (2011). A Novel Halophilic Lipase, LipBL, Showing High Efficiency in the Production of Eicosapentaenoic Acid (EPA) PLoS ONE; 6(8): e23325.

Phukon, L. C., Chourasia, R., Kumari, M., Godan, T. K., Sahoo, D., Parameswaran, B., & Rai, A. K. (2020). Production and characterisation of lipase for application in detergent industry from a novel *Pseudomonas helmanticensis* HS6. *Bioresource technology*, *309*, 123352.

Pimentel, M.C., Krieger, N., Coelho, L.C., Fontana, J.O and Melo, E.H. (1994). Lipase from a Brazilian strain of *Penicillium citrinum*. *Applied Biochemistry and Biotechnology*, 49: 59 -74.

Pogaku, P., Suresh, A., Srinivaslu, P and Reddy, S.A. (2010).Optimization of lipase production by *Staphylococcus* sp. Lp12. *African Journal of Biotechnology*, 9: 882- 886.

Pokorny, D., Friedrich, J and Cimerman, A. (1994). Effect of nutritional factors on lipase biosynthesis by *Aspergillus niger*. *Biotechnology Letters*, 16:363-366.

Polakovič, M., Vrabel, P., & Báleš, V. (1998). Approaches for improved identification of mechanisms of enzyme inactivation. In *Progress in Biotechnology* (Vol. 15, 77-82).

Prabaningtyas, R. K., Putri, D. N., Utami, T. S., & Hermansyah, H. (2018). Production of immobilized extracellular lipase from *Aspergillus niger* by solid state fermentation method using palm kernel cake, soybean meal, and coir pith as the substrate. *Energy Procedia*, *153*, 242-247.

Pradeep, S., Josh, M. S., Binod, P., Devi, R. S., Balachandran, S., Anderson, R. C., & Benjamin, S. (2015). *Achromobacter denitrificans* strain SP1 efficiently remediates di (2-ethylhexyl) phthalate. *Ecotoxicology and environmental safety*, *112*, 114-121.

Prajapati, V., Patel, H., Trivedi, U., & Patel, K. (2014). Kinetic and thermodynamic characterization of lipase produced by *Cellulomonas flavigena* UNP3. *Journal of basic microbiology*, 54(9), 976-983.

Pratt J, Cooley JD, Purdy CW, Straus DC. (2000). Lipase activity from strains of *Pasteurella multocida*, *Current Microbiology*, Vol. 40, 306-309.

Pratuamgdejkul J, Dharmsthiti S. (2000). Purification and characterization of lipase form psychrophilic *Acinetobacter calcoaceticus* LP009, *Microbiological Research*, Vol. 155, 95-100.

Priya KU, Reddy BI. (2015). Isolation, Optimization and Partial purification of Lipase Enzyme. *Biotechnol Appl Biochem* 6: 2156-2171.

Priyanka, P., Kinsella, G., Henehan, G. T., & Ryan, B. J. (2019). Isolation, purification and characterization of a novel solvent stable lipase from *Pseudomonas reinekei*. *Protein expression and purification*, *153*, 121-130.

Priyanka, P., Kinsella, G., Henehan, G.T., Ryan, B.J. (2019). Isolation, purification and characterization of a novel solvent stable lipase from *Pseudomonas reinekei*. *Protein expression and purification.* 153, 121-130.

Quivey RG, Faustoferri RJ, Monahan K, Marquis R. (2000). Shifts in membrane fatty acid profiles associated with acid adaptation of *Streptococcus* mutans. FEMS *Microbiology Letters,*189: 89–92

Rabbani, M., Shafiee, F., Shayegh, Z., MirMohammadSadeghi, H., Shariat, Z. S., Etemadifar, Z., & Moazen, F. (2015). Isolation and characterization of a new thermoalkalophilic lipase from soil bacteria. *Iranian Journal of Pharmaceutical Research: IJPR*, *14*(3), 901.

Rahman, R.N.Z.R., Baharum, S.N., Basri, M. and Salleh, A.B. (2005). High-yield purification of an organic solvent-tolerant lipase from *Pseudomonas* sp. strain S5. *Analytical biochemistry*, 341(2), 267-274.

Ramakrishnan, V., Goveas, L. C., Suralikerimath, N., Jampani, C., Halami, P. M., & Narayan, B. (2016). Extraction and purification of lipase from *Enterococcus faecium* MTCC5695 by PEG/phosphate aqueous-two phase system (ATPS) and its biochemical characterization. *Biocatalysis and agricultural biotechnology*, *6*, 19-27.

Ramani, K., Chockalingam, E and Sekaran, G. (2010). Production of a novel extracellular acidic lipase from *Pseudomonas gessardii* using slaughterhouse waste as a substrate. *Journal of Industrial Microbiology and Biotechnology,* 37: 531-535.

Rao JK, Argos P. (1981). Structural stability of halophilic proteins. Biochemistry 20(23):6536–6543.

Rashid, F. A. A.; Rahim, R. A.; Ibrahim, D.; Balan, A.; Bakar, N. M. A (2013). Purification and Properties of Thermostable Lipase from a Thermophilic Bacterium, *Bacillus licheniformis* IBRL-CHS2. JOURNAL OF PURE AND APPLIED MICROBIOLOGY 2013, 7, (3), 1635-1645.

Rashid, N., Shimada, Y., Ezaki, S., Atomi, H., & Imanaka, T. (2001). Low-temperature lipase from psychrotrophic *Pseudomonas* sp. strain KB700A. *Applied and Environmental Microbiology*, 67(9), 4064-4069.

Rasmey, A. H. M., Aboseidah, A. A., Gaber, S., & Mahran, F. (2017). Characterization and optimization of lipase activity produced by *Pseudomonas monteilli* 2403-KY120354 isolated from ground beef. *African Journal of Biotechnology*, *16*(2), 96-105.

Rathi, P., Goswami, V. K., Sahai, V., & Gupta, R. (2002). Statistical medium optimization and production of a hyperthermostable lipase from *Burkholderia cepacia* in a bioreactor. *Journal of applied microbiology*, *93*(6), 930-936.

Rathi, P., Sapna, B., Sexena, R., Gupta, R. (2000). A hyperthermostable, alkaline lipase from *Pseudomonas* sp. with the property of thermal activation. *Biotechnology Letters,* 22, 495–498.

Rathi, P., Saxena, R. K., & Gupta, R. (2001). A novel alkaline lipase from *Burkholderia cepacia* for detergent formulation. *Process Biochemistry*, *37*(2), 187-192.

Ray, A. K., Bairagi, A., Ghosh, K. S., & Sen, S. K. (2007). Optimization of fermentation conditions for cellulase production by *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 isolated from fish gut. Acta Ichthyologica et Piscatoria, 1(37), 47-53.

Reetz MT, Jaeger KE. (1998). Overexpression, immobilization and biotechnological application of *Pseudomonas* lipases, *Chemistry and Physics of Lipids*, Vol. 93, 3-14.

Reis P, Holmberg K, Watzke H, Leser ME, Miller R.(2009). Lipases at interfaces: a review. *Advances in colloid and interface science*;147–148:237–50.

Rigo E, Ninowa JL, Luccio MD, Oliveira JV, Polloni A, Remonatto D, Arbter F, Vardanega R, Oliveira DD, Treichel H. (2010). Lipase production by solid fermentation of soybean meal with different supplements, *Food Science and Technology*, Int., Vol. 43, 1132-1137.

Rivera-Utrilla, J., Ocampo-Pérez, R., Méndez-Díaz, J. D., & Sánchez-Polo, M. (2012). Environmental impact of phthalic acid esters and their removal from water and sediments by different technologies–a review. *Journal of Environmental Management*, 109, 164-178.

Rmili, F., Hadrich, B., Chamkha, M., Sayari, A., & Fendri, A. (2021). Optimization of an organic solvent-tolerant lipase production by *Staphylococcus capitis* SH6. Immobilization for biodiesel production and biodegradation of waste greases. *Preparative Biochemistry & Biotechnology*, 1-15.

Rodriguez, J. A., Mateos, J. C., Nungaray, J., González, V., Bhagnagar, T., Roussos, S.,& Baratti, J. (2006). Improving lipase production by nutrient source modification using *Rhizopus homothallicus* cultured in solid state fermentation. *Process Biochemistry*, 41(11), 2264-2269.

Rogalska, E., Cudrey, C., Ferrato, F. and Verger, R. (1993). Chirality 5, 24–30

Rohit, S., Chisti, Y and Uttam, C.B. 2001. Production, purification, characterization and applications of lipases. *Biotechnology Advance*, 19: 627-662.

Romdhane, I. B.-B.; Fendri, A.; Gargouri, Y.; Gargouri, A.; Belghith, H. (2010). A novel thermoactive and alkaline lipase from *Talaromyces thermophilus* fungus for use in laundry detergents. *Biochemical Engineering Journal*, 53, (1), 112-120.

Rooney, D and Weatherley, L. R. (2001). The effect of reaction conditions upon lipase catalyzed hydrolysis of high oleate sunflower oil in a stirred liquid-liquid reactor, *Process Biochemistry*, 36: 947-953.

Royter, M., Schmidt, M., Elend, C., Höbenreich, H., Schäfer, T., Bornscheuer, U.T. and Antranikian, G. (2009). Thermostable lipases from the extreme thermophilic anaerobic bacteria *Thermoanaerobacter thermohydrosulfuricus* SOL1 and *Caldanaerobacter subterraneus* subsp. tengcongensis. *Extremophiles*, 13(5), 769-783.

Rua ML, Dannert CS, Wahl S, Sprauer A, Schmid RD. (1997). Thermophilic lipase of *Bacillus thermocatenulatus* Large-scale production, purification and properties: aggregation behaviour and its effect on activity, *Journal of Biotechnology*, Vol. 56, 89-102.

Rua, M.L., Diaz-Maurino, T., Fernandez, V.M., Otero, C and Ballesteros, A. (1993). Purification and characterization of two distinct lipases from *Candida cylindracea*. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1156:181–189.

Rubin, B and Dennis, E.A. (1997a), Lipases: Part A. Biotechnology Methods in Enzymology. (Eds) Rubin, B and Dennis, E.A., vol., 284. New York: Academic Press, 1–408.

Rubin, B and Dennis, E.A. (1997b), Lipases: Part B. Enzyme characterization and utilization methods in enzymology. (Eds) Rubin, B and Dennis, E.A., vol. 286., New York: Academic Press, 1-563.

Ruchi G, Anshu G, Khare SK. (2008). Lipase from solvent tolerant *Pseudomonas aeruginosa* strain: Production optimization by response surface methodology and application, *Bioresource Technology*, Vol. 99, 4796-4802.

Ruiz C, Pastor FI, Diaz P. (2005). Isolation of lipid- and polysaccharide degrading microorganisms from subtropical forest soil and analysis of lipolytic strain *Bacillus* sp. CR-179, *Letter in Applied Microbiology*, Vol. 40, 218-227.

Russell, N. J. (1990). Cold adaptation of microorganisms. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, *326*(1237), 595-611.

Sachan, S., & Singh, A. (2015). Lipase enzyme and its diverse role in food processing industry. *Everyman's Sci*, *4*, 214-218.

Saengsanga, T., Siripornadulsil, W., & Siripornadulsil, S. (2016). Molecular and enzymatic characterization of alkaline lipase from *Bacillus amyloliquefaciens* E1PA isolated from lipid-rich food waste. *Enzyme and microbial technology*, *82*, 23-33.

Saengsanga, T., Siripornadulsil, W., & Siripornadulsil, S. (2016). Molecular and enzymatic characterization of alkaline lipase from *Bacillus amyloliquefaciens* E1PA isolated from lipid-rich food waste. *Enzyme and microbial technology*, *82*, 23-33.

Sagar, K., Bashir, Y., Phukan, M. M., & Konwar, B. K. (2013). Isolation of lipolytic bacteria from waste contaminated soil: A study with regard to process optimization for lipase. *Int. J. Sci. Technol. Res*, *2*(10), 214-218.

Salameh M, Wiegel J. (2007). Lipases from extremophiles and potential for industrial applications. *Advance in Applied Microbiology*, 61:253–283.

Salihu, A and Zahangir, M.A. (2012). Production and applications of microbial lipases: A review, Scientific Research and Essays. 7(30): 2667-2677.

Salis, A.; Bhattacharyya, M. S.; Monduzzi, M.; Solinas, V. (2009). Role of the support surface on the loading and the activity of *Pseudomonas fluorescens* lipase used for biodiesel synthesis. *Journal of Molecular Catalysis B: Enzymatic*, 57, (1), 262-269.

Salwoom, L., Raja Abd Rahman, R. N. Z., Salleh, A. B., Convey, P., Pearce, D., & Mohamad Ali, M. S. (2019). Isolation, characterisation, and lipase production of a cold-adapted bacterial strain *Pseudomonas* sp. LSK25 isolated from Signy Island, Antarctica. *Molecules*, *24*(4), 715.

Salwoom, L., Salleh, A. B., Convey, P., & Mohamad Ali, M. S. (2019). New recombinant cold-adapted and organic solvent tolerant lipase from psychrophilic *Pseudomonas* sp. LSK25, isolated from Signy Island Antarctica. *International journal of molecular sciences*, *20*(6), 1264.

Sangeetha, R., Arulpandi, I and Geetha, A. (2011). Bacterial lipases as potential industrial biocatalyst: An overview. *Research Journal of Microbiology*, 6(1): 1-24.

Sankar, K., & Achary, A. (2020). Bio-ceramic, mesoporous cuttlebone of Sepia officinalis is an ideal support for the immobilization of *Bacillus subtilis* AKL13 lipase: optimization, adsorption, thermodynamic and reaction kinetic studies. *Chemical Papers*, *74*(2), 459-470.

Saranya, P., Kumari, H. S., Rao, B. P., & Sekaran, G. (2014). Lipase production from a novel thermo-tolerant and extreme acidophile *Bacillus pumilus* using palm oil as the substrate and treatment of palm oil-containing wastewater. *Environmental science and pollution research*, 21(5), 3907-3919.

Sarkar P, Yamasaki S, Basak S, Bera A, Bag PK. (2012). Purification and characterization of a new alkali-thermostable lipase from *Staphylococcus aureus* isolated from Arachis hypogaea rhizosphere. *Process Biochemistry*, 47:858-866.

Sarkar S, Sreekanth B, Kant S, Banerjee R, Bhattacharyya BC. (1998). Production and optimization of microbial lipase, *Bioprocess Engineering*, Vol. 19, 29-32.

Sarkar, J., Chowdhury, P. P., & Dutta, T. K. (2013). Complete degradation of di-n-octyl phthalate by *Gordonia* sp. strain Dop5. *Chemosphere*, *90*(10), 2571-2577.

Sathyavrathan, P and Jaya, S. (2013). Production of lipase and protease from *Pseudomonas fragi* NRRL-B727 and optimization studies of the protease without the lipase presence. *International Journal of Chemical Technology and Research*, 5(4): 1541-1544.

Satti, S.M., Abbasi, A.M., Marsh, T.L., Auras, R., Hasan, F., Badshah, M., Farman, M. and Shah, A.A. (2019). Statistical optimization of lipase production from *Sphingobacterium* sp. strain S2 and evaluation of enzymatic depolymerization of poly (lactic acid) at mesophilic temperature. *Polymer Degradation and Stability*, *160*, 1-13.

Satyanarayana, T., Raghukumar, C., & Shivaji, S. (2005). Extremophilic microbes: Diversity and perspectives. *Current science*, 78-90.

Saunders, N. F., Thomas, T., Curmi, P. M., Mattick, J. S., Kuczek, E., Slade, R., & Cavicchioli, R. (2003). Mechanisms of thermal adaptation revealed from the genomes of

the Antarctic Archaea *Methanogenium frigidum* and *Methanococcoides burtonii*. *Genome research*, *13*(7), 1580-1588.

Saxena, R. K., Sheoran, A., Giri, B., & Davidson, W. S. (2003). Purification strategies for microbial lipases. *Journal of microbiological methods*, *52*(1), 1-18.

Sazci, A., Erenler, K., & Radford, A. (1986). Detection of cellulolytic fungi by using Congo red as an indicator: a comparative study with the dinitrosalicyclic acid reagent method. *Journal of Applied Bacteriology*, *61*(6), 559-562.

Scandurra, R., Consalvi, V., Chiaraluce, R., Politi, L., & Engel, P. C. (1998). Protein thermostability in extremophiles. *Biochimie*, *80*(11), 933-941.

Schmidt, C., Sztajer, H., Stocklein, W., Menge, U., Schimid, R. (1994). Screening, purification and properties of a thermophilic lipase from *Bacillus thermocatelnulatus*. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 1214, 43–53.

Schmidt-Dannert, C. (1999). Recombinant microbial lipases for biotechnological applications. *Bioorganic & medicinal chemistry*, 7(10), 2123-2130.

Schreier, P. (1997). Biotechnology of aroma compounds. Berger RG (ed.). *Advance in Biochemical Engineering and Biotechnology*, 17, 52.

Seeliger, H. P. (1956). Use of a urease test for the screening and identification of *cryptococci*. *Journal of Bacteriology*, *72*(2), 127.

Segel, I. H. (1976). Enzymes. Biochemical calculations, 208-323.

Sethi, B.K., Nanda, P.K., Sahoo, S. (2016). Characterization of biotechnologically relevant extracellular lipase produced by *Aspergillus terreus* NCFT 4269.10. *Brazilian Journal of Microbiology*, 47, 143–149.

Shabtai, Y and Daya-Mishne, N. (1992). Production, purification and properties of lipase from a bacetrium (*Pseudomonas aeruginosa* YS-7) capable of growing in water restricted environments. *Applied Environment and. Microbiology*, 58: 174-180.

Shafei, HAE, Rezkallah LA. (1997). Production, purification and characterization of *Bacillus* lipase, *Microbiological Research*, Vol. 152, 199-208.

Shailaja, S., Mohan, S.V., Krishna, M.R., Sarma, P.N. (2008). Degradation of di-ethylhexyl phthalate (DEHP) in bioslurry phase reactor and identification of metabolites by HPLC and MS. *International Biodeterioration and Biodegradation*. 62 (2), 143–152

Shailu Dalal, R. P. (2012). Bacterial Degradation of Phenol an Cyanide from Industrial Wastewater. *Bioremedial of Pollutants*, 1-23.

Shankar, T., & Isaiarasu, L. (2011). Cellulase production by *Bacillus pumilus* EWBCM1 under varying cultural conditions. *Middle-East Journal of Scientific Research*, *8*(1), 40-45.

Sharma C.K., Kanwar, S.S and Chauhan G.S. (2011). Synthesis of medically important ester ethyl cinnamate by porcein pancreatic lipase immobilized on Poly (AAc-co-HPMAcl-EGDMA) hydrogel, *Journal of Applies Polymer Science*, 121: 2674-2679.

Sharma, A. K., Tiwari, R. P., & Hoondal, G. S. (2001). Properties of a thermostable and solvent stable extracellular lipase from a *Pseudomonas* sp. AG-8. *Journal of Basic Microbiology: An International Journal on Biochemistry, Physiology, Genetics, Morphology, and Ecology of Microorganisms*, *41*(6), 363-366.

Sharma, A., Meena, K. R., & Kanwar, S. S. (2018). Molecular characterization and bioinformatics studies of a lipase from *Bacillus thermoamylovorans* BHK67. *International journal of biological macromolecules*, *107*, 2131-2140.

Sharma, P., Sharma, N., Pathania, S., & Handa, S. (2017). Purification and characterization of lipase by *Bacillus methylotrophicus* PS3 under submerged fermentation and its application in detergent industry. *Journal of Genetic Engineering and Biotechnology*, *15*(2), 369-377.

Sharma, R., Chisti, Y., & Banerjee, U. C. (2001). Production, purification, characterization, and applications of lipases. *Biotechnology advances*, *19*(8), 627-662.

Sharma, R., Soni, S., Vohra, R., Gupta, L., Gupta, J. (2002). Purification and characterization of a thermostable alkaline lipase from a new thermophilic *Bacillus* sp. RSJ-1. *Process Biochemistry*, 37, 1075–1084

Sharma, S.; Kanwar, S. S. (2014). Organic solvent tolerant lipases and applications. The Scientific World Journal.

Sharon, C., Furugoh, S., Yamakido, T., Ogawa, H and Kato, Y. (1998). Purification and characterization of a lipase from *Pseudomonas aeruginosa* KKA-5 and its role in castor oil hydrolysis. *Journal of industrial microbiology and biotechnology*, 20:304–307

Shimada, Y., Koga, C., Sugihara, A., Nagao, T., Takada, N., Tsunasawa, S and Tominaga, Y. (1993). Purification and characterization of a novel solvent tolerant lipase from *Fusarium heterosporum*, *Journal of Fermentation Bioengineering*, 75: 349- 352.

Shu, C., Xu, C and Lin, G. (2006). Purification and partial characterization of a lipase from *Antrodia cinnamomea*, *Process Biochemistry*, 41: 734 -738.

Shuler, M.L and Kargi, F. (2002). Bioprocess engineering basic concepts. 2nd edn. Prentice Hall, NJ. U.S.A. Chapter 5, 133.

Siddiqui, K. S., & Cavicchioli, R. (2006). Cold-adapted enzymes. *Annual Review Biochemistry.*, *75*, 403-433.

Sidhu P, Sharma R, Soni SK, Gupta JK. (1998a) Production of extracellular alkaline lipase by a new thermophilic *Bacillus* sp., *Folia Microbiologica*. Vol. 43, 51-54.

Sidhu, P., Sharma, R., Soni, S.K and Gupta, J.K. (1998b). Effect of cultural conditions on extracellular lipase production by *Bacillus* sp. RS-12 and its characterization. Indian *Journal of Microbiology*, 38:9-12.

Sierra G. (1957). A simple method for the detection of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. Antonie van Leeuwenhoek, 23:15 - 22.

Sifour, M., Zaghloul,T.I., Saeed,H.M., Berekaa, M.M and Abdelfattah, Y. R. (2010). Enhanced production of lipase by the thermophilic *Geobacillus stearothermophilus* strain-5 using statistical experimental designs. *New Biotechnology*, 27(4):330-336.

Siglioccolo A, Paiardini A, Piscitelli M, Pascarella S. (2011). Structural adaptation of extreme halophilic proteins through decrease of conserved hydrophobic contact surface. BMC *Structural Biology*, 11:50.

Sikora, A.; Siodmiak, T.; Marszall, M. (2014). Kinetic Resolution of Profens by Enantioselective Esterification. Chirality, 26, 663-669.

Silva, B. F., & Marques, E. F. (2005). Thermotropic behavior of asymmetric chain length catanionic surfactants: the influence of the polar head group. *Journal of colloid and interface science*, *290*(1), 267-274.

Silva, L. H. M and Meirelles, A. J. A. (2001). Phase equilibrium in polyethylene glycol/maltodextrin aqueous two-phase systems. *Carbohydrate Polymers*, 42: 273 278.

Simons JW, Adams H, Cox RC, Dekker N, Gotz F, Slotboom AJ, Verheij HM. (1996). The lipase from *Staphylococcus aureus* Expression in *Escherichia coli*, largescale purification and comparison of substrate specificity to *Staphylococcus hyicus* lipase, *European Journal of Biochemistry*, Vol. 242, No. 3, 760-769.

Simons, J.W., van Kampen, M.D., Riel, S., Gotz, F., Egmond, M.R and Verhey, H.M. (1998). Cloning, purification and characterization of the lipase from *Staphylococcus epidermidis* comparison of the substrate selectivity with those of other microbial lipases. *European Journal of Biochemistry*, 253:675-683.

Sinchaikul, S., Sookkheo, B., Phutrakul, S., Pan, F.M. and Chen, S.T. (2001). Optimization of a thermostable lipase from *Bacillus stearothermophilus* P1: overexpression, purification, and characterization. *Protein expression and purification*, 22(3), 388-398.

Singh, A. K., & Mukhopadhyay, M. (2012). Overview of fungal lipase: a review. *Applied biochemistry and biotechnology*, 166(2), 486-520.

Singh, M., Saurav, K., Srivastava, N., & Kannabiran, K. (2010). Lipase production by *Bacillus subtilis* OCR-4 in solid state fermentation using ground nut oil cakes as substrate. *Current Research Journal of Biological Sciences*, *2*(4), 241-245.

Singh, N., Dalal, V., Mahto, J.K., Kumar, P. (2017). Biodegradation of phthalic acid esters (PAEs) and in silico structural characterization of mono-2-ethylhexyl phthalate (MEHP)

hydrolase on the basis of close structural homolog. *Journal of Hazardous Materials*, 338 (15), 11–22.

Sirisha E, Rajaseker N, Narasu ML. (2010). Isolation and Optimization of lipase producing Bacteria from oil contaminated soils. *Advances in Biological research* 4: 249-252.

Sivaramakrishnan, R., & Incharoensakdi, A. (2016). Purification and characterization of solvent tolerant lipase from *Bacillus* sp. for methyl ester production from algal oil. *Journal of bioscience and bioengineering*, 121(5), 517-522.

Snellman, E. A., Sullivan, E. R., & Colwell, R. R. (2002). Purification and properties of the extracellular lipase, LipA, of *Acinetobacter* sp. RAG-1. *European Journal of Biochemistry*, *269*(23), 5771-5779.

Solanki, S.; Pandey, C. M.; Soni, A.; Sumana, G.; Biradar, A. M. (2016). An amperometric bienzymatic biosensor for the triglyceride tributyrin using an indium tin oxide electrode coated with electrophoretically deposited chitosan-wrapped nanozirconia. Microchimica Acta, 183, (1), 167-176.

Sonne, D. P.; Vilsbøll, T.; Knop, F. K. (2015). Pancreatic amylase and lipase plasma concentrations are unaffected by increments in endogenous GLP-1 levels following liquid meal tests. Diabetes care, 38, (5), e71-e72.

Srimhan P, Kongnum K, Taweerodjanakarn S, Hongpattarakere T. (2011). Selection of lipase producing yeasts for methanol-tolerant biocatalyst as whole cell application for palm-oil transesterification. *Enzyme and Microbial Technology*, 48: 293–298

Staple, J. K., Osen-Sand, A., Benfenati, F., Pich, E. M., & Catsicas, S. (1997). Molecular and functional diversity at synapses of individual neurons in vitro. *European Journal of Neuroscience*, *9*(4), 721-731.

Stead R (1986). Microbial Lipases their characteristics, role in food spoilage & industrial uses. *Journal of Dairy Research*, 53: 481-505.

Stepaniak, L., & Fox, P. F. (1983). Thermal stability of an extracellular proteinase from *Pseudomonas fluorescens* AFT 36. *Journal of Dairy Research*, 50(2), 171-184.

Stergiou, P. Y., Foukis, A., Filippou, M., Koukouritaki, M., Parapouli, M., Theodorou, L. G.& Papamichael, E. M. (2013). Advances in lipase-catalyzed esterification reactions. *Biotechnology advances*, *31*(8), 1846-1859.

Su, E.; Xu, J.; You, P (2014). Functional expression of *Serratia marcescens* H30 lipase in *Escherichia coli* for efficient kinetic resolution of racemic alcohols in organic solvents. *Journal of Molecular Catalysis B: Enzymatic*, 106, 11-16.

Sugihara, A., Scnoo, T., Enoki, A., Shimada, Y., Nagao, T and Tominaga, Y. (1995). Purification and characterization of a lipase from *Pichia burtonii*. *Applied Microbiology and Biotechnology*, 43:277-281.

Sugihara, A., Tani, T., & Tominaga, Y. (1991). Purification and characterization of a novel thermostable lipase from *Bacillus* sp. *The Journal of Biochemistry*, 109(2), 211-216.

Sun, J., Wu, X., & Gan, J. (2015). Uptake and metabolism of phthalate esters by edible plants. *Environmental Science & Technology*, *49*(14), 8471-8478.

Sun, J.; Yu, B.; Curran, P.; Liu, S.-Q. (2012). Lipase-catalysed transesterification of coconut oil with fusel alcohols in a solvent-free system. *Food Chemistry*, 134, (1), 89- 94.

Svendsen, A., Borch, K., Barfoed, M., Nielsen, T. B., Gormsen, E., & Patkar, S. A. (1995). Biochemical properties of cloned lipases from the *Pseudomonas* family. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 1259(1), 9-17.

Sztajer, H., Lunsdorf, H., Erdman, H., Menge, U and Schmid, R. (1992). Purification and properties of lipase from *Penicillium simplicissimum*, *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 1124: 253–261.

Takaç S, Marul B. (2008). Effects of lipidic carbon sources on the extracellular lipolytic activity of a newly isolated strain of *Bacillus subtilis*, *Journal of Indian Microbiology and Biotechnology*, Vol. 35, 1019-1025.

Takamoto, T., Shirasaka, H., Uyama, H and Kobayashi, S. (2001). Lipase-catalyzed hydrolytic degradation of polyurethane in organic solvent. Chemistry Letters.6:492 - 493.

Talon R, Dublet N, Montel MC, Cantonnet M. (1995). Purification and characterization of extracellular *Staphylococcus warneri* lipase, *Current Microbiology*, Vol. 30, 11-16.

Tambe, A., Vyasarayani, R., Datla, A., Ponrathnam, S., & Demnerova, K. (2015). Macroporous poly (vinyl acetate-co-divinyl benzene) copolymer beads as adsorptive support for the direct immobilization of *candida antarctica* lipase B. *Enzyme Eng*, 4, 2.

Tan S, Owusu ARK, Knapp J. (1996). Low temperature organic phase biocatalysis using cold-adapted lipase from psychrotrophic *Pseudomonas* P38. *Food Chemistry*, 57: 415-418.

Tan, T., Zhang, M., Wang, B., Ying, C and Deng, L. (2003). Screening of high lipase production *Candida* sp. and production of lipase by fermentation. *Process Biochemistry*, 39: 459-465.

Tanaka T, Ono E, Takinami K. (1981). Method of producing improved glyceride by lipase, United States Patent 4,275,011.

Tang, W. J., Zhang, L. S., Fang, Y., Zhou, Y., & Ye, B. C. (2016). Biodegradation of phthalate esters by newly isolated *Rhizobium* sp. LMB-1 and its biochemical pathway of di-n-butyl phthalate. *Journal of applied microbiology*, 121(1), 177-186.

Taskin, M., Ucar, M.H., Unver, Y., Kara, A.A., Ozdemir, M., Ortucu, S. (2016). Lipase production with free and immobilized cells of cold-adapted yeast *Rhodotorula glutinis* HL25. *Biocatalytic and Agricultural Biotechnology*, 8, 97-103.

Teather, R. M., & Wood, P. J. (1982). Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Applied and environmental microbiology*, *43*(4), 777-780.

Teymouri, M., Karkhane, M., Gilavand, F., Akhtari, J., & Marzban, A. (2018). Extracellular lipase purification from a marine *Planomicrobium* sp. MR23K and productivity optimization in a pilot-scale submerged bioreactor. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, *88*(2), 739-746.

Thakur, A., Kumar, A., & S Kanwar, S. (2012). Production of n-propyl cinnamate (musty vine amber flavor) by lipase catalysis in a non-aqueous medium. *Current Biotechnology*, *1*(3), 234-240.

Thakur, S. (2012). Lipases, its sources, properties and applications: a Review. Int J Sci Eng Res 3, 1-29.

Thomson, C.A., Delaquis, P.J and Mazza, G. (1999). Detection and measurement of microbial lipase activity: A review. *Critical Review in Food Science and Nutrition*, 39(2): 165- 187.

Toida, J., Kondoh, K., Fukuzawa, M., Ohnishi, K., & Sekoguchi, J. (1995). Purification and characterization of a lipase from *Aspergillus oryzae*. *Bioscience, biotechnology, and biochemistry*, *59*(7), 1199-1203.

Torres S, Castro GR. (2004). Non-aqueous biocatalysis in homogeneous solvent systems. *Food Technology and Biotechnology*, 2:271–7.

Tran, D. T., Lin, Y. J., Chen, C. L., & Chang, J. S. (2013). Kinetics of transesterification of olive oil with methanol catalyzed by immobilized lipase derived from an isolated *Burkholderia* sp. strain. *Bioresource technology*, *145*, 193-203.

Treichel, H., de Oliveira, D., Mazutti, M. A., Di Luccio, M., & Oliveira, J. V. (2010). A review on microbial lipases production. *Food and bioprocess technology*, *3*(2), 182-196.

Turati, D.F.M., Almeida, A.F., Terrone, C.C., Nascimento, J.M., Terrasan, C.R., Fernandez Lorente, G., Carmona, E.C. (2019). Thermotolerant lipase from *Penicillium* sp. section Gracilenta CBMAI 1583: Effect of carbon sources on enzyme production, biochemical properties of crude and purified enzyme and substrate specificity. *Biocatalytic and Agricultural Biotechnology,* 17, 15-24.

Tyndall, J.D.A., Sinchaikul, S., Fothergill Gilmore, L.A., Taylor, P., Walkinshaw, M.D. (2002). Crystal structure of a thermostable lipase from *Bacillus stearothermophilus* P1, *Journal of Molecular Biology*, 323: 859–869.

Undurraga, D., Markovits, A., & Erazo, S. (2001). Cocoa butter equivalent through enzymic interesterification of palm oil midfraction. *Process biochemistry*, *36*(10), 933-939.

Unni, K. N., Priji, P., Sajith, S., Faisal, P. A., & Benjamin, S. (2016). *Pseudomonas aeruginosa* strain BUP2, a novel bacterium inhabiting the rumen of Malabari goat, produces an efficient lipase. Biologia, 71(4), 378-387.

Uttatree, S., Winayanuwattikun, P., & Charoenpanich, J. (2010). Isolation and characterization of a novel thermophilic-organic solvent stable lipase from *Acinetobacter baylyi*. *Applied biochemistry and biotechnology*, *162*(5), 1362-1376.

Vakhlu, J and Kour, A. (2006). Yeast lipases: Enzyme purification, biochemical properties and gene cloning, *European Journal of Biotechnology*, 9(1):69–81.

Van Den Burg, B. (2003). Extremophiles as a source for novel enzymes. *Current opinion in microbiology*, *6*(3), 213-218.

Vaseghi, Z., Najafpour, G. D., Mohseni, S., Mahjoub, S., & Hosseinpour, M. N. (2012). Lipase production in tray-bioreactor via solid state fermentation under desired growth conditions. *Iranica Journal of Energy and Environment*, *3*(1), 59-65.

Vasiee, A., Behbahani, B. A., Yazdi, F. T., & Moradi, S. (2016). Optimization of the production conditions of the lipase produced by *Bacillus cereus* from rice flour through Plackett-Burman Design (PBD) and response surface methodology (RSM). *Microbial pathogenesis*, *101*, 36-43.

Veerapagu, M., Narayanan, A. S., Ponmurugan, K., & Jeya, K. R. (2013). Screening selection identification production and optimization of bacterial lipase from oil spilled soil. *Asian J. Pharm. Clin. Res*, *6*(3), 62-67.

Villeneuve, P., Muderhwa, J.M., Graille, J and Haas, M.J. (2000). Customizing lipases for biocatalysis: A survey of chemical, physical and molecular biological approaches. *Journal of Molecular Catalysis B: Enzymatic*, 9:113-148.

Voget, Sonja, Andreas Knapp, Anja Poehlein, Christel Vollstedt, Wolfgang Streit, Rolf Daniel, and Karl-Erich Jaeger. "Complete genome sequence of the lipase producing strain *Burkholderia glumae* PG1." *Journal of biotechnology* 204 (2015): 3-4.

Wagner, A., & Daum, G. (2005). Formation and mobilization of neutral lipids in the yeast Saccharomyces cerevisiae. *Biochemical Society Transactions*, 33(5), 1174-1177.

Wakelin NG, Forster CF. (1997). An investigation into microbial removal of fats, oils and greases, *Bioresource Technology*, Vol. 59, 37-43.

Wang YX, Srivastava KC, Shen GJ, Wang HY. (1995). Thermostable alkaline lipase from a newly isolated thermophilic *Bacillus*, strain A30-1 (ATCC53841), *Journal of Fermentation Bioengineering*, Vol. 79, 433-438.

Wang, B., Wang, A., Cao, Z., & Zhu, G. (2016). Characterization of a novel highly thermostable esterase from the Gram-positive soil bacterium *Streptomyces lividans* TK64. *Biotechnology and applied biochemistry*, *63*(3), 334-343.

Wang, Q., Hou, Y., Ding, Y., & Yan, P. (2012). Purification and biochemical characterization of a cold-active lipase from Antarctic sea ice bacteria *Pseudoalteromonas* sp. NJ 70. *Molecular biology reports*, *39*(9), 9233-9238.

Wang, W.X., Fan, C.Q. (2014). Gas/solid particulate phthalic esters (PAEs) in Masson pine (*Pinus massoniana L*.) needles and rhizosphere surface soils. *Journal of Hazardous Materials*, 276, 149–156.

Wang, W.X., Xu, X.B., Fan, C.Q. (2015). Health hazard assessment of occupationally di-(2- ethylhexyl)-phthalate-exposed workers in China. *Chemosphere* , 120, 37–44.

Wang, W., Zhang, Y., Wang, S., Fan, C. Q., & Xu, H. (2012). Distributions of phthalic esters carried by total suspended particulates in Nanjing, China. Environmental monitoring and assessment, 184(11), 6789-6798.

Wei Y, Swenson L, Castro C, Derewenda U, Minor W, Arai H, Aoki J, Inoue K, Gonzalez LS, Derewenda ZS. (1998), Structure of a microbial homologue of mammalian platelet-activating factor acetylhydrolases: *Streptomyces exfoliatus* lipase at 1.9 Å resolution, Structure, Vol. 511-519.

Wen, Z.W., Wu, W.M., Ren, N.Q., Gao, D.W. (2016). Synergistic effect using vermiculite as media with a bacterial biofilm of *Arthrobacter* sp. for biodegradation of di-(2-ethylhexyl) phthalate. *Journal of Hazardous Materials*, 304, 118–125

Whitaker, J. R., & Stauffer, C. E. (1994). Principles of Enzymology for the Food Sciences (2nd edn). *Trends in Food Science and Technology*, *5*(9), 304-304.

Wiegel, J. (1998). Anaerobic alkalithermophiles, a novel group of extremophiles. *Extremophiles*, *2*(3), 257-267.

Wiegert, T., Homuth, G., Versteeg, S., & Schumann, W. (2001). Alkaline shock induces the *Bacillus subtilis*σW regulon. *Molecular microbiology*, 41(1), 59-71.

Wilson, K. (2001). Preparation of genomic DNA from bacteria. *Current protocols in molecular biology*, 56(1), 2-4.

Wiseman A. (1995). Introduction to principles. In: Wiseman, A. editor. Handbook of enzyme biotechnology. 3rd ed. Padstow, Cornwall, UK: Ellis Horwood Ltd. T.J. Press Ltd., 3−8.

Woese, C., Sogin, M., Stahl, D., Lewis, B. J., & Bonen, L. (1976). A comparison of the 16S ribosomal RNAs from mesophilic and thermophilic *Bacilli*: some modifications in the Sanger method for RNA sequencing. *Journal of Molecular Evolution*, *7*(3), 197-213.

Woolley, P and Peterson, S. B. (1994). Lipases-their structure, biochemistry and applications. Press, Cambridge: Cambridge University.

Wu, H. S., & Tsai, M. J. (2004). Kinetics of tributyrin hydrolysis by lipase. *Enzyme and microbial technology*, *35*(6-7), 488-493.

Xin, J. Y., Li, S. B., Xu, Y., Chui, J. R., & Xia, C. G. (2001). Dynamic enzymatic resolution of naproxen methyl ester in a membrane bioreactor. Journal of Chemical Technology & Biotechnology: International Research in Process, *Environmental & Clean Technology*, 76(6), 579-585.

Xin, Li, Hui-Ying, Yu., (2012). Characterization of a novel extracellular lipase from a halophilic isolate, *Chromohalobacter* sp. LY7-8. *African Journal of Microbiology Research*, 6, 3516-3522.

Xu, D., Deng, X., Fang, E., Zheng, X., Zhou, Y., Lin, L.,& Huang, Z. (2014). Determination of 23 phthalic acid esters in food by liquid chromatography tandem mass spectrometry. *Journal of Chromatography* A, 1324, 49-56.

Xu, G., Li, F., & Wang, Q. (2008). Occurrence and degradation characteristics of dibutyl phthalate (DBP) and di-(2-ethylhexyl) phthalate (DEHP) in typical agricultural soils of China. *Science of the Total Environment*, *393*(2-3), 333-340.

Xu, J., Lu, Q., de Toledo, R. A., & Shim, H. (2017). Degradation of di-2-ethylhexyl phthalate (DEHP) by an indigenous isolate *Acinetobacter* sp. SN13. *International Biodeterioration & Biodegradation*, 117, 205-214.

Xu, X. R., Li, H. B., & Gu, J. D. (2005). Biodegradation of an endocrine-disrupting chemical di-n-butyl phthalate ester by *Pseudomonas fluorescens* B-1. *International Biodeterioration & Biodegradation*, 55(1), 9-15.

Xu, X., Li, H., Gu, J., Li, X. (2007). Kinetics of n-butyl benzyl phthalate degradation by a pure bacterial culture from the mangrove sediment. *Journal of Hazardous Material*, 140 (1), 194e199.

Yadav, R.P., Saxena, R.K., Gupta, R and Davidson, S. (1998). Lipase production by *Aspergillus* and *Penicillium* species. *Folia Microbiologica*., 43(4): 373-378

Yamanashi, T., Kim, S. Y., Hara, H., & Funa, N. (2015). In vitro reconstitution of the catabolic reactions catalyzed by PcaHG, PcaB, and PcaL: the protocatechuate branch of the β-ketoadipate pathway in *Rhodococcus jostii* RHA1. *Bioscience, biotechnology, and biochemistry*, *79*(5), 830-835.

Yamne T, Iwasaki Y, Roxana R, Shimidzu N and Doisaki N. (2002). Multiple intensified performance of an enzyme-catalyzed reaction in organic medium. Ann NY Acad Sci 22: 43-50.

Yan, J., Han, B., Gui, X., Wang, G., Xu, L., Yan, Y., Madzak, C., Pan, D., Wang, Y., Zha, G. and Jiao, L. (2018). Engineering *Yarrowia lipolytica* to simultaneously produce lipase and single cell protein from agro-industrial wastes for feed. *Scientific reports*, *8*(1), 1-10.

Yang, J., Guo, D., & Yan, Y. (2007). Cloning, expression and characterization of a novel thermal stable and short-chain alcohol tolerant lipase from *Burkholderia cepacia* strain G63. *Journal of Molecular Catalysis B: Enzymatic*, 45(3-4), 91-96.

Yang, T., Ren, L., Jia, Y., Fan, S., Wang, J., Wang, J., & Yan, Y. (2018). Biodegradation of di-(2-ethylhexyl) phthalate by *Rhodococcus ruber* YC-YT1 in contaminated water and soil. International journal of environmental research and public health, 15(5), 964.

Yang, W., He, Y., Xu, L., Zhang, H., & Yan, Y. (2016). A new extracellular thermo-solvent-stable lipase from *Burkholderia ubonensis* SL-4: Identification, characterization and application for biodiesel production. *Journal of Molecular Catalysis B: Enzymatic*, *126*, 76-89.

Yang, X., Wang, B., Cui, F and Tan, T. (2005). Production of lipase by repeated batch fermentation with immobilized *Rhizopus arrhizus*. *Process Biochemistry*, 40: 2095-2103.

Ye, L.; Zhang, B.; Seviour, E. G.; Tao, K.-x.; Liu, X.-h.; Ling, Y.; Chen, J.-y.; Wang, G. (2011). Monoacylglycerol lipase (MAGL) knockdown inhibits tumor cells growth in colorectal cancer. *Cancer letters*, 307, (1), 6-17.

Yoo H-Y, Simkhada JR, Cho SS, Park DH, Kim SW, Seong CN, Yoo JC. (2011). A novel alkaline lipase from *Ralstonia* with potential application in biodiesel production. *Bioresource Technology*, 102: 6104–6111.

Yue, F. A. N. G., Zhang, L., Jing, W. A. N. G., Ying, Z. H. O. U., & Bangce, Y. E. (2017). Biodegradation of phthalate esters by a newly isolated *Acinetobacter* sp. strain LMB-5 and characteristics of its esterase. *Pedosphere*, 27(3), 606-615.

Zaks, A., & Klibanov, A. M. (1988). The effect of water on enzyme action in organic media. *Journal of Biological Chemistry*, 263(17), 8017-8021.

Zarevúcka, M. (2012). Olive oil as inductor of microbial lipase Olive Oil-Constituents, Quality, Health Properties and Bioconversions, InTech Europe, Rijeka, Croatia, 457-470.

Zhang, H., Lin, Z., Liu, B., Wang, G., Weng, L., Zhou, J., Hu, H., He, H., Huang, Y., Chen, J. and Ruth, N. (2020). Bioremediation of di-(2-ethylhexyl) phthalate contaminated red soil by *Gordonia terrae* RL-JC02: Characterization, metabolic pathway and kinetics. *Science of the Total Environment*, *733*, 139138.

Zhang, J., Zhang, C., Zhu, Y., & Li, X. (2018). Biodegradation of seven phthalate esters by *Bacillus mojavensis* B1811. *International Biodeterioration Biodegradation* S0964830518300829.

Zhao, H. M., Du, H., Lin, J., Chen, X. B., Li, Y. W., Li, H., & Wong, M. H. (2016). Complete degradation of the endocrine disruptor di-(2-ethylhexyl) phthalate by a novel *Agromyces* sp. MT-O strain and its application to bioremediation of contaminated soil. *Science of the total Environment*, 562, 170-178.

Zhao, H. M., Hu, R. W., Huang, H. B., Wen, H. F., Du, H., Li, Y. W., & Wong, M. H. (2017). Enhanced dissipation of DEHP in soil and simultaneously reduced bioaccumulation of DEHP in vegetable using bioaugmentation with exogenous bacteria. *Biology and Fertility of Soils*, 53(6), 663-675.

Zhao, H.M., Du, H., Lin, J., Chen, X.B., Li, Y.W., Li, H., Cai, Q.Y., Mo, C.H., Wong, M.H. (2016a). Complete degradation of the endocrine disruptor di-(2-ethylhexyl) phthalate by a novel *Agromyces* sp. MT-O strain and its application to bioremediation of contaminated soil. *Science of Total Environment*, 562, 170–178.

Zhao, H.M., Hu, R.W., & Chen, X.X. (2018). Biodegradation pathway of di-(2-ethylhexyl) phthalate by a novel *Rhodococcus pyridinivorans* XB and its bioaugmentation for remediation of DEHP contaminated soil. *Science of Total Environment*, 640, 1121–1131.

Zhao, H.M., Hu, R.W., Huang, H.B., Wen, H.F., Du, H., Li, Y.W., Li, H., Cai, Q.Y., Mo, C.H., Liu, J.S. and Wong, M.H., (2017). Enhanced dissipation of DEHP in soil and simultaneously reduced bioaccumulation of DEHP in vegetable using bioaugmentation with exogenous bacteria. *Biology and Fertility of Soils*, 53(6), 663-675.

Zhao, J., Ma, M., Zeng, Z., Yu, P., Gong, D., & Deng, S. (2021). Production, purification and biochemical characterisation of a novel lipase from a newly identified lipolytic bacterium *Staphylococcus caprae* NCU S6. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *36*(1), 248-256.

Zheng, X., Chu, X., Zhang, W., Wu, N., & Fan, Y. (2011). A novel cold-adapted lipase from *Acinetobacter* sp. XMZ-26: gene cloning and characterisation. *Applied microbiology and biotechnology*, *90*(3), 971-980.

Zheng-Yu, S. H. U., Jiang-Ke, Y. A. N. G., & Yun-Jun, Y. A. N. (2007). Purification and characterization of a lipase from *Aspergillus niger* F044. *Chinese Journal of* Biotechnology, 23(1), 96-101.

Zhou, J., Chen, W., Jia, Z., Huang, G., Hong, Y., Tao, J., & Luo, X. (2012). Purification and characterization of lipase produced by *Aspergillus oryzae* CJLU-31 isolated from waste cooking oily soil. *American journal of food technology*, 7(10), 596-608.

Zottig, X., Meddeb-Mouelhi, F., Charbonneau, D. M., & Beauregard, M. (2017). Characterization of a Novel Alkalophilic Lipase From *Aneurinibacillus thermoaerophilus*: Lid Heterogeneity and Assignment to Family I. 5. *The protein journal*, 36(6), 478-488.

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Biochemical characterization and thermodynamic study of lipase from psychrotolerant *Pseudomonas punonensis*

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ABSTRACT

A psychrotolerant lipolytic strain *Pseudomonas punonensis* was isolated from soil of cold desert of Leh (Laddakh) India. This strain was first time screened for production of extracellular lipase enzyme. The present study aimed for the production, partial purification, biochemical characterization, kinetic and thermodynamic parameters of lipase from this novel strain. Study of physico-chemical characteristic of lipase enzyme showed maximum activity at 30 °C and pH 9.0. Arrhenius plot of temperature dependent enzyme activity revealed the activation energy as 27.74 kJ/mol and the temperature quotient was calculated to be 1.01. The kinetic constants of the enzyme such as V_{max}, and K_m derived from Lineweaver- Burk plot of the enzyme activity recorded at 30 °C, were found to be 86.22 U mL^{-1} and 0.163 mM respectively. Experiment on thermal inactivation of enzyme showed thermal inactivation energy ($E_{a(d)}$) to be 56.89 kJ/mol. Thermodynamic values of lipase were found as Gibb's free energy (87.44 kJ/mol), enthalpy (54.29 kJ/mol) and entropy (-0.109 kJ/mol/K) for the enzyme denaturation obtained at 30 °C. The thermodynamic values suggested that enzyme catalysis was spontaneous and efficient at 30 °C when compared with 50 and 70 °C.

1. Introduction

Enzymes are mainly used as biocatalyst in a variety of industrial applications. In industry, there is always a high demand for novel enzymes, which provide better assets for specific applications in industrial activities (Konarzycka-Bessler and Jaeger, 2006). Microbial enzymes produced by extremophilic microorganisms, often exhibit stability and activity in water as well as in the presence of different types of organic solvents. These microbial enzymes are specifically important for bioconversion of substrate that is slightly soluble or insoluble in water. Lipolytic enzymes are the most important biocatalysts for playing important functions in both aqueous and nonaqueous media (Karadzic et al., 2006). Lipase (EC 3.1.1.3) belong to a special class of hydrolase which not only catalyzes the hydrolysis of triglyceride into glycerol and fatty acids, but also performs *trans*-esterification and esterification as well. Lipases belongs to serine hydrolases group, defined as triacylglycerol acyl hydrolases (E.C. 3.1.1.3) which are different from esterase (E.C. 3.1.1.1) enzymes by their substrates.. Lipase producing microorganisms are found in various habitats such as oil-contaminated soil, industrial waste of oil mills, dairy waste, oil seeds and putrefying food and coal tips and hot spring. Microbial lipases can be both extracellular and intracellular or periplasmic, and are strongly influenced by physicochemical and nutritional conditions, such as temperature, pH of culture media, carbon and nitrogen sources, inorganic salts etc. (Gupta et al., 2004).

Although, most of the bacterial lipases are produced by *Pseudomonas* genus and among them, few species are well-known psychrophilic bacteria (Rajmohan et al., 2002). Some *pseudomonas* species are the producers of commercial lipases such as *Pseudomonas alcaligenes, Pseudomonas cepacia,* and *Pseudomonas mendocina* etc. Chigusa et al. (1996) and Wang et al. (2009) suggested that extracellular lipase mainly produced by *Pseudomonas* and *Bacillus* strains are among the first studied lipolytic enzymes with potential applications in the field of biotechnology.

Generally, the extracellular lipase enzyme is inducible enzyme, which is generally produced in the presence of inducers, but its production is induced by triacylglycerol, vegetable oils, oil industry wastes, surfactants and salts (Damaso et al., 2008). Lipases have great potential in the medical and pharmaceutical industry, formation of flavored compounds in food industry, detergent and leather industry and synthesis of heat-labile compounds. Besides these industrial applications, it is also useful in biotechnological industry for making biopolymer,

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ORIGINAL PAPER

Kinetic and thermodynamic characterization of novel alkaline lipase from halotolerant *Bacillus gibsonii*

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Abstract

A halotolerant bacterial strain isolated and identified as *Bacillus gibsonii* was used for extracellular lipase production. The bacterial strain was able to grow up to 1200 mM salt concentration and showed maximum growth at 600 mM NaCl concentration. The present study includes production of extracellular lipase enzyme and characterization of partially purified lipase with respect to its kinetic and thermodynamic behaviour. Maximum lipase activity was observed at 60 °C under alkaline pH (9.0) condition. The kinetic parameters such as V_{max}, K_m and K_{cat} were calculated as 158.73 U/mL, 0.539 mM and 483.93 min^{-1} at 60 °C, respectively, suggested thermostable nature of the enzyme. The thermal inactivation energy [$E_{a(d)}$] was calculated as 66.98 kJ/mol. The values of Gibb's free energy (86.31 kJ/mol), enthalpy (64.26 kJ/mol) and entropy (-66.21×10^{-3} kJ/mol/K) for the enzyme inactivation obtained at 60 °C corroborated the assumption that 60 °C was the optimum temperature. Further, the deactivation rate constant (k_d) values calculated at 60 °C and 80 °C were found to be 0.0907 and 0.182 min^{-1}, respectively, which suggested that enzyme was more stable at 60 °C and it was partly inactivated at 80 °C.

Keywords Extracellular lipase · Halotolerant · Kinetic and thermodynamic property · Thermal stability · Thermal inactivation

Introduction

Lipases are defined as triacylglycerol acylhydrolases (EC 3.1.1.3) which naturally hydrolyze triglycerides into diglycerides, monoglycerides, glycerol and fatty acids (Houde et al. 2004). Microbial lipases have some additional properties, which make them potent enzyme for esterification, transesterification, aminolysis, alcoholysis, and deacetylation reactions (Sarmah et al. 2018). Microbial lipases, not only hydrolyze ester bonds of triglycerides, but also resolve racemic mixture and synthesize ester bonds in non-aqueous media (Hiol et al. 1999). Lipolytic activity in bacteria is dependent on the presence of carbon source as well as inducers such as oil, fatty acids, bile salts and triacyl glycerols (Bora and Mohan 2008). Microbial lipases produced by mesophilic microorganisms are active over a wide range of alkaline pH conditions, but are generally unstable at temperatures above 70 °C. Microbial lipases produced by mesophilic bacteria are generally more active between 30 and 65 °C (Royter et al. 2009). The thermostable microbial lipases have been purified and characterized from *Bacillus* sp. (Sugihara et al. 1991), *Bacillus thermocatenulatus* (Schmidt-Dannert et al. 1996), *B. stearothermophilus* (Kim et al. 2000), *Bacillus* sp. J33 (Nawani and Kaur 2000), *B. strain* A30-1 (Wang et al. 1995), *Bacillus* sp. RSJ-1 (Sharma et al. 2002) and *B. thermoleovorans* ID-1 (Lee et al. 1999).

Since microbial lipases are mostly used in industries at elevated temperatures, it is pertinent to study their thermal behaviour. Discovery of new thermo-stable microbial lipases have attracted the attention of many researchers with respect to developing strategies to enhance stability of lipases at elevated temperature. A major constraint in achieving the thermal stability of enzyme is the kinetic limitations imposed by high temperature, they require more rigid material, which are unsupportive to product formation (Li and Zhang 2005). The importance of thermostable lipases has been growing as they are more stable than the mesophilic lipase, which make them more potent for biotechnological applications. Thermostable lipase

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