Characterization of thermostable and alkalophilic lipase enzyme from endophytic fungus *Leptosphaerulina sp*

A Dissertation
Submitted in partial fulfillment of the requirement
For the award of degree of
Masters of Science in Biotechnology

Under the guidance of
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Head & Professor

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CERTIFICATE

This is to certify that the thesis entitled **"Characterization of thermostable and alkalophilic lipase enzyme from endophytic fungus *Leptosphaerulina sp*"** submitted by **Kirti Singh** (Roll no. 301101017) in partial fulfillment of the requirement for the award of Degree of Master of Sciences in Biotechnology, to Thapar University, Patiala is a record of student's own work carried out by her under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

15.7.13

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DECLARATION

I hereby declare that the work which is being presented in this thesis **"Characterization of thermostable and alkalophilic lipase from endophytic fungus *Leptosphaerulina sp*"** submitted by the undersigned in partial fulfillment of the requirement for the award of Degree of Master of Sciences in Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of **Dr. MS Reddy,** Head & Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree.

Date: July 15, 2013

Place: Patiala **(Kirti Singh)**

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And above all, I bow my head to the Almighty for his blessings.

Kirti Singh

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LIST OF ABBREVIATIONS

Gly-X-Ser-X-Ser	Glycine-X-Serine-X-Serine
GRAS	Generally regarded as safe
EDTA	Ethylene diamine tetracetic acid
FDA	Food and Drug Administration
kDa	Kilo Dalton
U/ml	Units/ml
mM	millimolar
w/v	weight/volume
v/v	volume/volume
U/mg	Units/mg
C-endophytes	Clavicipitatious-endophytes
NC-endophytes	Non Clavicipitatious-endophytes
MRA	Mycelium Radiens Astrovivens
CPG	Controlled porous glass
SDS	Sodium dodecyl Sulphate
SSF	Solid state fermentation
POC	Pongamia oil cake
U/g DM	Units/gram Dry Matter
CDB	Czapek Dox Broth
PDA	Potato Dextrose Agar
PDB	Potato dextrose Broth

psi	Per square inch
pNPP	para Nitrophenyl palmitate
pNP	para Nitrophenol
pNPA	p para Nitrophenyl Acetate
µM	micromolar
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
BSA	Bovine serum albumin
µg/ml	microgram/ml

CHAPTER 1

INTRODUCTION

1.1 Lipase

The large part of the earth's biomass is represented by lipids. Lipids are essential to all living systems. They are the most important source of energy, play structural roles in membranes and are involved in signalling events. To be able to carry out these functions, lipids require lipolytic enzymes during their metabolism. Lipolytic enzymes catalyze the turnover of these water-insoluble compounds [Gilham and Lehner 2005]. They also breakdown lipids and make them mobile within the cells of individual organisms [Beisson *et al*. 2000]. Lipolytic enzymes are grouped into 3 main categories, which are esterases, phospholipases and lipases [Arpigny and Jaeger 1999].

Lipases were first discovered in 1856 by Claude Bernard when he studied the role of the pancreas in fat digestion [Peterson and Drablos 1994]. Since then, many different lipases have been identified in and isolated from bacteria, fungi, plants, and animals. In 1958, lipases were first defined by Sarda and Desnuelle based on the phenomenon of interfacial activation and they concluded that the activity of lipase was related to the formation of interface between the water-insoluble substrate and water [Verger 1997].

Human pancreatic lipase and *Rhizomucor miehei* lipase were the first ones whose 3D structures were elucidated in 1990. These two lipases were found to have a lid like structure covering the active site of the enzyme and the presence of such a lid might be a good explanation for interfacial activation of the enzyme. It was postulated that this structure had to show a change in conformation in the presence of lipid-water interface and this might be related to the activation of enzymes [Frenken *et al*. 1992]. Finally, it was clearly identified that both lipases were active via the movement of lid that closes the active site since the active site of the enzyme became accessible to the substrate. However some lipases with exceptional 3D structures from *Pseudomonas glumae, Pseudomonas aeruginosa* and *Candida antartica* have a lid structure, but do not show interfacial activation to be active [Schmid and Verger 1998; Verger 1997].

Therefore, lipases are defined basically as fat-splitting enzymes that catalyze hydrolysis of long-chain triacylglycerols to form glycerol and fatty acid in the presence of excess water (Figure 1).

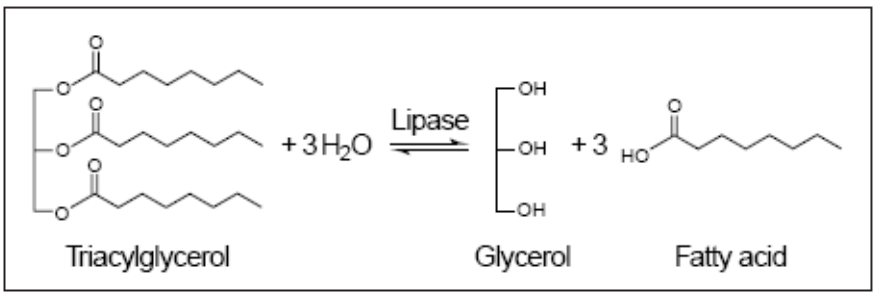


Figure 1: Hydrolytic and Synthetic Actions of Lipase
[Source: Jaeger and Reetz 1998]

Also, they can catalyze the reverse reaction, synthesis of triacylglycerols, under non-aqueous conditions [Jaeger *et al*. 1999; Gupta *et al*. 2004; Pascale *et al*. 2008]. Glycerolesters with an acyl chain length ≥ 10 carbon atoms can be said as lipase substrates [Jensen 1983]. In addition to lipases, esterases are also grouped into hydrolases and these two enzymes were confused for a long time. Now, it is realized that they are really different from each other in terms of substrate specificity [Zhang and Zeng 2008]. Esterases break ester bonds of short chain fatty acids whereas lipases catalyze the hydrolysis of long chain fatty acids that are insoluble or poorly soluble. Therefore, the lipase must be capable of identifying an insoluble or aggregated substrate.

1.2 General Lipase Structure

It is very important to know 3D structures of lipases in order to make them fit for specific applications. Human pancreatic lipase and the lipase from the fungus *Rhizomucor miehei* were the first ones whose 3D structures elucidated. Various other fungal lipase structures from *Geotrichum candidum*, *Fusarium solani*, *Candida rugosa*, *Candida antarctica*, *Humicola lanuginose* and *Rhizopus delemar* followed them [Jaeger *et al*. 1999; Jaeger *et al*. 1994].The first bacterial lipase structure from *Pseudomonas glumae,* was clarified in 1993 [Noble *et al*. 1993].

After the determination of first lipase structures, it was realized that they share a common folding pattern called α/β hydrolase fold in spite of not showing sequence similarity [Fan *et al*. 2008]. They compared five hydrolytic enzymes which were dienelactone hydrolase, haloalkane dehalogenase, wheat serine carboxypeptidase II, acetylcholinesterase and the lipase from *Geotrichum candidum*. They concluded that they share a same folding pattern because they all catalyze a hydrolysis reaction [Arpigny and Jaeger 1999; Nardini and Dijkstra 1999]. So the alpha/beta-hydrolase fold family consists of structurally related enzymes with diverse catalytic functions.

The α/β hydrolase fold includes a central, mostly parallel eight- stranded β sheet (only the

second β strand is antiparallel) surrounded on both sides by α helices (Figure 2).The β sheet displays a left-handed superhelical twist and the first and the last strands cross each other at an angle of approximately 90° [Jaeger *et al*. 1999]. The number of β strands in β sheet can be changeable for lipases from different species.

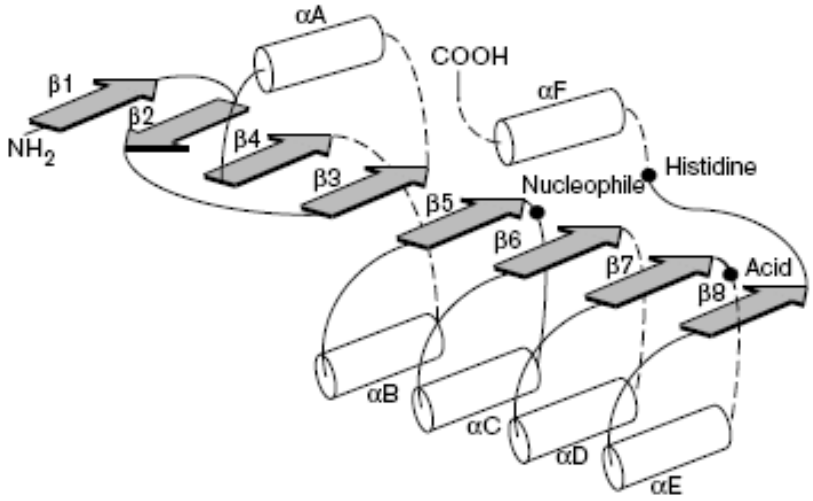


Figure 2: The a/ß hydrolase fold
[Source: Jaeger *et al*. 1999]

The active site of the α/β hydrolase fold enzymes contains three catalytic residues which are nucleophilic residues (serine, cysteine or aspartate), a catalytic acid residue (aspartate or glutamate) and a histidine residue. In lipases the nucleophile residue has been determined to be a serine residue, but the catalytic acid can be either an aspartate or a glutamate residue. The active site serine residue is located in a highly conserved Gly-X-Ser-X-Ser pentapeptide [Pascale *et al*. 2008; Joseph *et al*. 2008].

Another unusual and interesting feature of the structure of most lipases is the presence of a lid-like structure that consists of one or two α-helices. This property results in a conformational change in lipase if there is an interface between oil and water (interfacial activation phenomenon). The lid moves away from the active site, thereby allowing it to become accessible for the substrate [Angkawidjaja and Kanaya 2006]. However exceptions are the lipases from *Pseudomonas glumae* and *Candida antartica*. In spite of having a lid, these lipases do not show interfacial activation [Schmid and Verger 1998]. Some *Pseudomonas* lipases are shown to have a calcium binding site. This calcium binding site is located near the active site, but it is not related to catalytic activity. It is thought to play a role in stabilization of the general structure of the enzyme.

1.3 Sources of Lipases

Lipases are found throughout all kingdoms of life, which are prokaryotes including bacteria and archaea and eukaryotes including plants, animals and fungi [Hong *et al*. 2008]. Microbial lipases are more useful than enzymes derived from plants and animals, since they have great variety of catalytic activities and microorganisms are easy to manipulate genetically and capable of rapid growth on inexpensive media. Furthermore, microorganisms are not affected by seasonal fluctuations so they can be supplied regularly and high amounts of lipases may be obtained from microbial cells. Microbial lipases are also more stable than their plant and animal derivatives and their production is easier and safer for industrial and research applications [Schmidt and Verger1998].

Aspergillus niger is one of the most important microorganisms used in biotechnology. It has already been in use for decades to produce many extracellular enzymes that are considered GRAS by the FDA (United States of America) [Schuster *et al.* 2002]. Fungal lipases have been studied since 1950's. These lipases are being exploited due to their low cost of extraction, thermal and pH stability, substrate specificity, and activity in organic solvents. Fungal lipases have benefits over bacterial ones due to the fact that present day technology favors the use of batch fermentation and low cost extraction methods. Fungal Lipase is a specific enzyme that digests fat and is characterized by its ability to hydrolyze fat over a wide range of temperatures and pH. Fungal Lipase is effective in regulating the levels of cholesterol and triglycerides and is also helpful in different dietary regime for weight management. The main producers of commercial lipases mainly are from fungal and yeast like *Aspergillus niger, Candida cylindracea, Humicola lanuginosa, Mucor miehei, Rhizopus arrhizus, Rhizopus delemer, Rhizopus japonica* and *Rhizopus oryzea* [Schuster *et al.* 2002]. Lipases from *Geotrichum candidum, Rhizopus* and *Aspergillus* strains are attractive catalysts for lipid modification.

1.4 Detection of lipolytic microorganisms

There must be three factors to detect a lipase-positive species by culturing it. These factors include (i) growth of the organism, (ii) production of lipase by that organism under suitable growth conditions and (iii) the presence of a sensitive method to detect lipase activity [Shelley *et al.* 1987].

Lipase activity is identified by using triacylglycerols composed of long-chain fatty acids. Triolein is the most ideal substrate due to its liquid form at common assay temperatures. This feature facilitates emulsification of it into growth media. Alternatively, olive oil can be used instead of triolein. Olive oil has the advantage of including high concentration of oleic acid and being more economical [Jensen 1983].

Table A: Assay for the determination of lipase activity [Gupta *et al*. 2003]

Assay and substrate	**Product analyzed**	**Principle involved**
Plate assays : Tributyrin, acyl glycerols and esters of long chain fatty acids	Short chain fatty acid	Halo based or colour change of Phenol red/ Victoria blue/ Sulphate or measurement of fluorescence after complexation of fatty and with fluorescent dye Rhodamine B
Titrimetry: Fats and oils, triacyl glycerols, methyl esters	Fatty acids	Neutralization reaction either directly by pH stat or by pH indicator
Spectrophotometry: Fatty acid conjugates of β napthol P-nitrophenyl esters	β napthol P nitrophenyl	Estimation of β napthol by complexation with fast blue BB coloured product measured at 410 nm.
Tweens	Fatty acid	Precipitation of fatty acid with calcium or copper & measurement of turbidity
Fluorescence assays: Triglycerols with alkyl group substituted with a fluorescent group	Fluorescent free phenyl group Fluorescent 4 methyl umbelliferone	Shift in fluorescence wavelength after triacylglycerol hydrolysis Product is analysed as it is fluorescent

1.5 Fermentation Conditions

Microbial lipases are mostly released outside of the cell that is these are extracellular enzymes. Microbial lipases are influenced by nutritional and physico-chemical factors; such as temperature, pH, nitrogen and carbon sources, presence of lipids, inorganic salts, stirring conditions, dissolved oxygen concentration [Rosenau and Jaeger 2000].

The major factor for the expression of lipase enzyme is carbon source. Lipases are generally produced in the presence of lipid source such as oil, triacylglycerols, fatty acids, hydrolyzable esters, tweens and glycerols. In addition to carbon source, the type of nitrogen source also influence the production of lipases. Generally, organic nitrogen source preferred by microbes are peptone and yeast extract [Gupta *et al*. 2004].

The initial pH of the growth medium is important for lipase production. Most microbes prefer pH around 7.0 for their best growth and lipase production. The optimum temperature for lipase production is parallel with the growth temperature of the respective microorganism.

1.6 Industrial Applications of Microbial Lipases

The global industrial enzyme market was about $ 2 billion in 2004 and is estimated to reach nearly $ 3.5 billion in 2012 with a year-on-year growth *rate* of 6.1%. Lipases represent about 4% of this market and thus has been paid an increasing attention due to their biotechnological potential [Hasan *et al*. 2006].

Lipases as biocatalysts have many favourable properties that make them suitable for specific applications when compared with chemical catalysts. Lipase-catalyzed reactions are highly specific because of their broad substrate specificity and high regio and/or stereoselective features while chemical processes are typically more non-specific. Due to the specificity of lipases, the production of unwanted products in the waste stream are decreased or eliminated. Moreover, the use of enzymes decreases the side reactions and make post-reaction separation problems simpler [Hasan *et al*. 2006].

The applications of lipases include:

- Organic syntheses
- Hydrolysis of fats & oils
- Modification of fats
- Flavor enhancement in food processing

- Resolution of racemic mixtures
- Chemical analysis

Lipases thus find a role in the following industries:

- Food & Dairy industry [cheese ripening, flavor development]
- Detergent industry
- Pharmaceutical industry
- Agrochemical industry
- Oleo chemical industry
- Leather industry

Apart from these, lipases are being involved in the field of clinical medicine and very importantly in research on malfunctions in lipid metabolism and the diseases of the circulatory and nervous system.

Microbial Lipases:

Microbial lipases may be extracellular, intracellular and membrane bound [Sharma and Banerjee 2001]

a) Extracellular:

Bacterial: *Chromobacterium viscosum, Pseudomonas nitroreducens, Pseudomonas aeruginosa, Proteus vulgaris*

Fungal: *Aspergillus niger, Penicillium* spp*, Mucor* spp*, Rhizopus arrhizus*

b) Intracellular: *Rhizopus oryzae, Rhizopus javanicus, R. chimensis*

c) Membrane bound*: Geotrichum candidum*

The lipases may be classified according to pH.

- Acidic***:*** *Aspergillus niger*
- Alkaline: *Pseudomonas fluorescens, Pseudomonas pseudoalcaligenes, Penicillium expansum.*

1.7 Microorganisms as Bio-Factories

The biosphere is occupied by a wide variety of microorganisms that carry out important functions like global primary energy and element cycling and they form the largest part of living organisms in the sense of total biomass cell numbers (6×10^{30} bacteria, 1.3×10^{28} archaea, 3.1×10^{29} eukarya), cell biomass (6×10^{18} kg bacteria, 1.3×10^{16} kg archaea and 3.1×10^{17} kg eukarya) and species diversity. This diversity of microorganisms is the most

common source of genes which can be used in several industrial and research applications [Beloqui *et al*. 2008].

Microbes are able to break down a variety of usual and unusual carbon and energy sources and convert them into amino acids, nucleotides, vitamins, carbohydrates and fatty acids by producing specific enzymes. Enzymes that carry out metabolic processes in microbes also have several practical and industrial usages to perform certain reactions apart from the cell. So, due to their ability to adapt a wide range of conditions and produce specific enzymes, microorganisms have been paid attention as little bio-factories [Sanchez 2005].

In the beginning of enzyme technology, crude preparations from certain animal tissues like pancreas and stomach mucosa, or plant tissues found applications in textile, leather and other industries. However, such preparations had some disadvantages such as high cost and shortage of tissues from animals and plants. It was realized that some microorganisms produce enzymes similar to that of plants and animals in terms of actions. Jokichi Takamine (1894) was the first person to introduce microbial enzymes to industry. Boidin and Effront (1917) were pioneers in the production of bacterial enzymes. Since that time microbial enzymes have taken the place of enzymes from plants and animals [Underkofler *et al*. 1957].

Microbial enzymes have a great usage in food, pharmaceutical, textile, paper, leather and other industries [Hasan *et al*. 2006]. Their applications have been increasing rapidly. Among industrially important enzymes, hydrolases come in the first place and include enzymes with a wide substrate specifity. Carbohydrases, proteases, pectinases and lipases are classified into hydrolases. These enzymes catalyze the hydrolysis of natural organic compounds [Underkofler *et al*. 1957].

1.8 Endophytic fungi

The fungi associated with plants are highly diverse, some of them are endophytes. The term fungal endophyte defines a fungus of which at least a significant part of its life cycle resides in a plant, and which colonizes tissues without causing symptoms of disease. Fungal endophytes can grow inter– and intra–cellulary as well as endo– and epi–phytically [Schulz and Boyle 2005]. They are not restricted to one environment but were detected in various surroundings including those with extreme characteristic.

Endophytic fungal communities adapt to different physiological conditions, in consequence they were detected in the wide spectrum of plant tissue types. Many neutral fungal endophytes are asleep pathogens which may be activated and cause infectious symptoms

when the host plant is aged and/or stressed. The endophytic microbial communities play an essential role in the physiology of host plants. Hosts colonized by endophyte, often have more vigor due to secretion of plant growth–promoting substances such as indole–3–acetic acid [Ek *et al*. 1983; Robinson *et al.* 1998] or cytokines [Crafts and Miller 1974], and improvement of the hosts absorption of nutritional nitrogen and phosphorus [Lyons *et al.* 1990]. Additionally, the endophyte partner can extensively enhance plants resistance to biotic and abiotic challenges [Latch 1993]. These beneficial features have been observed in infected plants exposed to several abiotic stresses such as drought [Cheplick *et al.* 2000], heavy metals [Monneta *et al*. 2001], high salinity [Waller *et al*. 2005].

They improve the resistance of host plants to adversity by secretion of bioactive metabolites. These metabolites are of unique structure including alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones and xanthones [Tan and Zou 2001]. They find wide-range of application in agrochemicals industries, antibiotics, immunosuppressants, antiparasitics antioxidants and anticancer agents [Gunatilaka *et al*. 2006]. Like other organisms invading plant tissues, endophytic fungi produce extracellular hydrolases as a resistance mechanism against pathogenic invasion and to obtain nutrition from host. Such enzymes include pectinases, cellulases [Caldwell *et al. 2000*], lipases [Petrini *et al.* 1992], laccase from the endophytic *Monotospora sp* [Wang *et al.* 2006], xylanase [Suto *et al.* 2002], α-1,4- glucan lyase [Nielsen *et al.* 1998], phosphotases [Maccheroni *et al.* 1998] and proteinase [Reddy *et al.* 1996*;* Lindstorm *et al.* 1994]. Caldwell *et al*. (2000) reported the ability of dark septate root endophytic fungi, *Philaophora finlandia* and *Phialocephela fortinii* isolated from alpine plant communities to breakdown major polymeric forms of carbon, nitrogen and phosphorus found in plants. Maria *et al.* (2005) studied the enzyme activity of endophytic fungi from mangrove angiosperm *Acanthus ilicifolius* L. and mangrove fern *Acrostichum aureum* L. of southwest coast of India. Choi *et al.* (2005) screened the endophytic fungi for their ability to produce lignocellulases, amylase, cellulase, ligninase, pectinase and xylanase. Although the mechanism is unclear, endophytic fungi actually played an important role in local ecology [Clay and Holah 1999; Omacini *et al.* 2001]. These qualitative assays help us in understanding whether fungi can change their mode of life from an endophyte, to a saprobe or pathogen.

The production of extracellular enzymes for penetration and limited colonization of selected plant cell [Gamboa and Bayman, 2001; Promputtha *et al.* 2005] is a common trait of

endophytic fungi. In literature, the main studies on endophytic fungi include screening for secondary metabolites, with antimicrobial and antioxidant activity. Not many have explored the possibility of endophytic fungi as biotechnological sources of industrially relevant enzymes. Hence they occupy a relatively unexplored site and can represent a source in obtaining different enzymes with potentialities.

We will focus on a thermostable and alkalophillic lipase from an endophytic fungus *Leptosphaerulina sp* in this particular study.

CHAPTER 2

REVIEW OF LITERATURE

Over the past few years endophytes have been recognized as the potential sources of novel natural products for exploitation in medicine, agriculture and industry. Enzymes like lipase, amylase, protease, ligninase, xylanase with potential use in industry can be readily identified in fungal endophytes.

Pokorny *et al.* (1996) isolated and characterized 2 different lipases from *Aspergillus niger* strain MZKI Al 16 in submerged culture. Lipase production was induced by addition of olive oil to a complex medium with an initial pH of 5.0. Maximal activity was reached after 70 h in a 151 bioreactor at 30°C with aeration of 0.5 vvm and agitation 400 rpm. Optimal temperature and pH conditions for the action of the lipases tested on tributyrin were 45°C and pH 7.0. Triacetin and tributyrin were shown to be the best substrates. The presence of iron and silver ions at low concentrations did not alter the activities. Both the enzymes possessing lipase activity were isolated by acetone precipitation followed by purification using ion-exchange chromatography. The molecular weights were 43 kDa and 65 kDa with isoelectric points of pH 4.1 and 4.2, respectively. The higher molecular weight lipase showed preference towards 1- and 3-positions of the triglyceride molecule and was stereoselective for the *sn*- 1 position with an enantiomeric excess of 20%. It displayed strong activity toward naphthyl, indolyl, umbelliferyl and resorufin esters and was active on esters of hydroxynaphthoic acid anilide, while it showed no activity towards esters of hydroxypyrene trisulfonic acid.

Research was carried out by Berto and Belingheri (1997) on lipase production in *Alternaria brassicicola* in which they found that higher quantity (3.2 U/ml) of an inducible extracellular lipase (EC 3.1.1.3) was produced in shaken synthetic medium which was supplemented with 20 mM methyloleate. After purification, the molecular weight of the lipase was determined 80 kDa by SDS-PAGE and estimated at 85 kDa using gel filtration, which suggested that the enzyme may be a monomer. The optimum pH and temperature for activity of the enzyme were 9.0 and 25ºC, respectively. Using umbelliferone esters, the lipase was shown to be highly specific towards a synthetic substrate with long-chain unsaturated fatty acid.

Ecological factors which affect the diversity of endophytes have been studied by Rajagopal & Suryanarayanan (2000). They also highlighted relationship between geographic locations and climatic variations. Occurrence and distribution of foliar endophytes of four different types of tropical forests found in India, viz. dry thorn forest, dry deciduous forest, moist deciduous forest and semi-evergreen forest of Western Ghats of Southern India have also been studied.

Huang *et al.* in (2001) concluded that taxonomically, most of the endophytic fungi belong to the phylum Ascomycota and its associated anamorphs, while some species belong to the phyla Basidiomycota and Zygomycota.

Suryanarayanan *et al.* (2002) focussed upon isolation and study of endophytic fungi from tree species, mangroves, palms and grasses. Different tissue types, viz. cotyledons, seed coats, stems, leaves and petiole were also screened to isolate endophytes. Although, endophytes from roots were considered differently, attempts have been made to isolate root endophytes from different hosts.

Moataza *et al.* (2004) optimized lipase production from a strain of *Fusarium oxy*sp*orum*. After optimization of culture conditions in liquid medium the highest lipase production amounting to 44.9 U/ml was achieved after 5^{th} day of cultivation at 28 ± 1°C. The medium was supplemented with 0.2% (w/v) peptone and sunflower oil was used as carbon source. Lipase activity was found to be optimum at pH 7.0 and 55°C and a good stability up to 90% activity retention was observed at pH ranging from 6.5 to 8.0. The enzyme activity rapidly dropped at temperature above 70°C. Analysis of enzymatic glycerolysis of triolein by thinlayer chomatography showed a clear fatty acid spot.

Bischoff & White (2005) classified endophytic fungi in 4 classes. C-endophytes (Class 1 endophytes) represent a small number of phylogenetically related clavicipitaceous species that are fastidious in culture and limited to some cool- and warm-season grasses. Typically these endophytes occur within plant shoots. Class 2 endophytes comprise a diversity of species, all of which are members of the Dikarya (Ascomycota or Basidiomycota) [Rayner *et al.* 1915]. Most belong to the Ascomycota, with a minority of Basidiomycota. Class 2 endophytes are distinct from the other NC-endophytes because in general they colonize roots, stems and leaves are capable of forming extensive infections within plants; are transmitted via seed coats and/or rhizomes; have low abundance in the rhizosphere; and

typically have high infection frequencies (90–100%) in plants growing in high-stress habitats. Class 3 endophytes are distinguished on the basis of their occurrence primarily or exclusively in above-ground tissues; horizontal transmission; the formation of highly localized infections. Class 3 endophytes include the hyperdiverse endophytic fungi associated with leaves of tropical trees [Lodge *et al*. 1996; Frohlich & Hyde 1999] as well as the highly diverse associates of above-ground tissues of nonvascular plants, seedless vascular plants, conifers. Merlin (1922) observed a brown to blackish, pigmented fungus associated with terrestrial plant roots. These sterile, root-associated fungi were called 'mycelium radicus astrovirens'(MRA) and were often found to co-exist with mycorrhizal fungi, and were referred to as 'pseudomycorrhizal' fungi angiosperms associated with dark pigmented fungi in root tissues [Peyronel 1924]. Presently, these fungi are referred to as 'dark septate endophytes' and categorized under class 4 endophytes.

Bancerz and Ginalska (2007) studied esterification properties of partially purified lipase from *Bjerkandera adusta* R59 which was immobilized on CPG and its properties were compared with those of the free enzyme. The free and immobilized lipases showed optimal activities at 45 and 50°C, respectively. Both enzyme forms were highly thermostable up to 60°C. The enzymes were stable at pH from 6.0 to 9.0 and their optimal pH for activity was 7.0. The free lipase was more thermostable in *n*-hexane than in aqueous environment. Both lipase preparations had good stabilities in non-polar solvents and were capable of hydrolyzing a variety of synthetic and natural fats. Non-immobilized lipase activity was inhibited by disulphide bond reagents, serine and thiol inhibitors, while EDTA and serine had no effect on enzyme activity. All anionic detergents tested in experiments inhibited lipase activity. The free lipase showed good stability in the presence of commercial detergents at laundry pH and temperatures.

Savitha *et al.* (2007) studied a total of 32 fungal organisms belonging to 3 different genera which were isolated from various sources of which 4 (three *Aspergillus sp* and one *Mucor sp*) were found to be positive for lipase production. Rhodamine B rapid screening technique was used to determine the lipase activity. *Mucor sp* was found to exhibit a maximum fluorescence zone at 350 nm. Among the various types of triglycerides used as the carbon source, sunflower oil was found to be the most effective in inducing lipase in *Mucor sp* .The lipase in *Mucor sp* was found to be inducible, alkalophilic and thermostable.

Ali *et al.* (2009) reported production of extracellular lipase by *Isaria fumosoroseus* isolate IF28.2 using different combinations of basal medium components. The effect of addition of different vegetable oils to the basal medium at different concentrations to improve enzyme production was evaluated. Maximum lipase activity (125.33 U/ml) as well as maximum biomass production (22.37mg/ml) was observed for olive oil when used at a concentration of 2% (v/v) of the basal medium. In the presence of surfactants, the highest lipase activity occurred when SDS and Tween 80 were added at the time of fungal inoculation. SDS proved to be the best surfactant having 110.66 U/mL lipase activity. The effects of the divalent metal ions (iron and magnesium) on lipase activity were also studied. Iron was found to inhibit, whereas magnesium slightly increased lipase activity. The optimum pH for lipase production was 5.7 while 32°C proved to be the best temperature for lipase production.

Balaji and Ebenezer (2008) cultivated *Colletotrichum gloeosporioides* for lipase production in SSF using oil-mill residue as a solid support. The elaboration of extracellular lipases from *C. gloeosporioides* on different residual cheap oil substrates was tested. Among these, POC showed maximum lipase activity 983 U/g DM followed by coconut oil cake (COC) 925 U/g DM. These two oil industrial residues were chosen for enhanced lipase production. The maximum enzyme yield was obtained when POC was impregnated with CDB in the ratio of 1:1.5 and with 906 U/g DM. Triton X 100 as the extractant from the fermented solids yielded 1020 U/g DM. The optimum temperature for lipase secretion by *C. gloeosporioides* was found to be 25°C. Xylose as the carbon source and peptone as the nitrogen source were favorable for the secretion of the enzymes. Tween 60 served as a good lipid substrate for lipase production (1170U/g DM) when POC was the substrate. Amending the POC with Magnesium sulphate as a metallic ion source induced good lipase activity (1240 U/g DM) by *C. gloeosporioides*. The Sunflower oil was found to be best co-vegetable oil substrate to induce the enzyme (2560 U/g DM). Triton X100 served as the surfactant for lipase secretion in *C.gloeosporioides*.

Shangguan *et al.* (2011) worked on a novel lipase gene from *Aspergillus fumigatus*, afl1-1, which was cloned and expressed with a molecular mass of 38 kDa in *Escherichia coli* for the first time. The recombinant lipase had a preference for short carbon chain p-NP esters, especially toward C2 p-NP ester and exhibited potent hydrolysis activity that had not been observed. The optimum pH and temperature of this new enzyme were 8.5 and 65 °C, respectively. The recombinant lipase (AFL1-1) was an alkaline enzyme which was stable in

the pH range 6.0-8.5 for 16 h (at 4°C) and at 30-50°C for 1 h. The Michaelis–Menten kinetic parameters V_{max} and K_m of the lipase were 1.37 mM mg^{-1} min^{-1} and 14.0 mM, respectively. Ca^{2+} and other metal ions could not activate the lipase. According to the homology analysis and site-directed mutagenesis assay, the catalytic triad of the recombinant lipase was identified as Ser-165, Asp-260, and His-290 residues.

A total of 36 fungal strains were isolated from oil contaminated soil collected from in and around Hyderabad by Thota *et al.* (2012). The fungal isolates were screened using Rhodamine-B agar media and Tributyrin Agar medium. Two of the isolated strains exhibited a greater clear zone than the others, indicating higher lipase activity. Therefore, these two strains (TP.St02 and TP.St05) were selected and identified based on their morphological and physiological characteristics. Phylogenetic analyses based on the results of 18S rDNA gene sequencing revealed that TP.St02 were close in identity to *Rhizopus niveus*. The optimum pH and temperature for lipase production by TP.St.02 were found to be 6.5 at 30°C, after 96 h of incubation, while TP.St05 were found to be 6.5 at 32°C, after 96 h of incubation. In addition, increased enzymatic production was obtained when the organisms were cultured in medium supplemented with 2% fructose as the carbon source. Among the different lipase inducers, both strains utilized Tween 80 and produced a great level of extracellular lipase.

Objectives

1. Optimization of growth conditions for lipase activity

2. Evaluation of optimum pH and temperature for maximum lipase activity

3. Evaluation of Carbon and Nitrogen sources for maximum lipase activity

CHAPTER 3

MATERIALS AND METHODS

Organism and culture conditions

Leptosphaerulina sp which isolated from a tropical semi-arid habitat subject to dry season fire in the Western Ghats, Southern India was used in this study.

The pure culture was maintained on PDA plates and PDB at 25°C under static conditions.

3.1 Qualitative screening of fungus for lipase production

Qualitative screening was done in order to confirm the production of lipase by fungal species using Tween 20 Agar media (Appendix 1-1.1). All the components were weighed and added to 100ml of distilled water.

1. Final pH of media was adjusted to 6.0 using 1N HCl.
2. Media was autoclaved for 15 minutes at 121°C at pressure of 15psi.
3. 1 ml of Tween 20 was filter sterilized and added to above media.
4. Finally the media was poured into petri plates and the fungus was subcultured on this media to check for zone of pecipitation around the fungus. [Jian *et al.* 1998]

3.2 Enzyme characterization

3.2.1 Growth medium and cultural conditions

All the components of Basal media (Appendix1- 1.2) were mixed and final volume was made 100 ml using distilled water in 250ml Erlenmeyer flask and was autoclaved at 15 psi for 15 minutes. These flasks were inoculated with the fungus and incubated at 28±1 °C in shaking incubator (80 rpm) and crude extract taken after desired incubation time [Tiwari *et al.* 2011].

3.2.2 Assay of lipase activity-colorimetric assay

1. Crude enzyme extract was prepared from the culture supernatant which was centrifuged at 10,000 rpm for 15 min at 4°C.

Lipase activity was determined with p-NPP by the method reported by *Licia et al*. (2006). In this method p-NPP was hydrolysed by lipase to give p-NP which gave yellow colour absorbance of which was measured spectrophotometrically at 410 nm.

2. The substrate for this reaction composed of solution A and solution B. Solution A contained 40 mg of p-NPP dissolved in 12 ml isopropanol. SolutionB contained 0.1 g of gum arabic and 0.4 ml of triton X-100 dissolved in 90 ml of distilled water.
3. The substrate solution was prepared by adding 1 ml of solution A to 19 ml of solution B drop wise with constant stirring to obtain an emulsion that remains stable for 2 h.
4. The assay mixture contained 1 ml of the substrate, 0.5 ml of buffer {Potassium phosphate buffer, pH 7, 0.1 M (Appendix 1-1.3)}, 0.1 ml of enzyme (the filtrate) and the volume was made up to 4 ml with distilled water.
5. This was incubated at 50ºC for 30 min.
6. The enzyme activity was stopped by adding 0.2 ml of isopropanol.
7. The absorbance was measured at 410 nm against enzyme free blank.
8. The standard graph was prepared by using p-NP (20-100 μM) in 2mM CAPS buffer (Appendix 1-1.4) pH 11 according to Table 1(Appendix 2) and absorbance taken at 410 nm.

3.2.3 Enzyme Activity Calculation

Enzyme activity (U/ml enzyme) was calculated acccording to equation below. The highest enzyme activity data was assumed 100% and relative enzyme activity was estimated for each data. To calculate specific enzyme activity (U/mg protein), bradford assay was applied. Specific enzyme activity was obtained by division of enzyme activity value to protein content (mg/ml). One lipase unit (U) is defined as the amount of enzyme that liberated 1 μM p-NP per min under the assay conditions described [Maia *et al.*1999].

Enzyme activity and specific activities were calculated as per the following formula:
U/ml Enzyme can be calculated as:

$$\frac{\mu M \text{ of p-NP released } * \times \text{Total reaction volume}}{\text{Vol. used in spectrophotometric determination} \times \text{Vol. of enzyme} \times \text{Incubation time}} \quad (1)$$

Where

Total reaction volume = 4 ml
Vol. used in spectrophotometric determination = 3ml
Vol. of enzyme = 0.1 ml
Incubation time = 30 minutes

*μM of p-NP released were calculated using straight line equation obtained from standard graph of p-NP as:

$$OD_{410} = 0.008 \times Conc.\ p\text{-}NP\ (\mu M) + 0.009$$

Hence

$$\text{Conc. p-NP } (\mu M) = \frac{OD_{410} - 0.009}{0.008} \quad (2)$$

$$\text{Specific activity (U/mg protein)} = \frac{\text{Enzyme activity (U/ml)}}{\text{Protein content (mg/ml)}} \quad (3)$$

3.3 Bradford Total Protein Assay

The Bradford total protein assay is the spectroscopic analytical method which is used to determine the total protein concentration of responsible sample. In this method, Coomassie brilliant blue G-250 dye binds to proteins and changes their colour from green to blue. That colour change is monitored at 595nm in UV-visible spectrophotometer. As the concentration of protein content is increase the colour gets darker. Coomassie brilliant blue G-250 binds to arginine, lysine, and histidine residues in protein samples [Bradford M.M 1976]. A series of BSA standard in different concentrations were prepared according to Table 2 (Appendix 2). According to this BSA standard, standard calibration curve was drawn with response to their absorbance values. Total protein content was calculated from standard calibration curve equation.

Preparation of protein sample:

- 1 ml of crude extract (protein) was diluted with 1 ml of distilled water.
- 3 ml of Bradford reagent (Appendix 1-1.3) was added
- Mixture was left for 5 minutes at room temperature
- Absorbance was taken at 595nm

3.4 Biochemical characterization of enzyme

3.4.1 Determination of Optimum Lipase Production Time

To increase the enzyme production fungal species was grown at 25°C under shaking (80 rpm) in 100 ml basal medium supplemented with 1% (v/v) olive oil as lipase substrate and

as a sole carbon source. Aliquots of the culture broth were withdrawn after two days interval and harvested (10,000g at 4°C for 15 min) to determine the optimum time for lipase production. The supernatants were used for measurement of lipase activity as per section 3.2.2 and 3.2.3.

3.4.2 Effect of glucose as additional Carbon Source

To monitor the effect of glucose, olive oil and their combination on the production of lipase, 4 ml of 20% (w/v) glucose solution, 1% (v/v) olive oil and both glucose and olive oil were added to 100 ml basal medium separately. Fungal species was inoculated and was grown on these three media at 25°C with shaking (80 rpm) for 5 days. After incubation, all cultures were harvested at 10,000 g for 15 min. The cell free supernatants were subjected to lipase assay section 3.2.2 and 3.2.3.

3.4.2 Effect of pH on lipase activity and stability

The supernatant which gave the highest lipase activity was selected and optimum pH and temperature tests were done with it.

In order to find the pH profiling of lipase activity, Sodium acetate buffer (pH 4-5), Potassium phosphate buffer (pH 6-7), Tris-HCl buffer (pH 8), Glycine-NaOH buffer (pH 9-12) were selected (Appendix 1-1.6). All buffers were of 0.1M concentration. The assay mixture (500 µl each buffer, 1 ml substrate and 100 µl enzyme solution) was incubated at 40°C for 30 minutes and enzyme activity determined by spectropohotometric assay using *p*-NPP as a substrate. The relative lipase activity was measured as a percentage (%) by comparison it with highest value.

3.4.3 Effect of temperature on the activity and stability of lipase

A temperature gradient was employed in order to determine the lipolytic activity of the enzyme. In this case 2 mM CAPS buffer pH 11 was used (Appendix 1-1.4). The buffer, substrate solution, and crude enzyme extract were incubated over range of temperatures between 30°C to 100°C for 30–60 minutes at the observed optimum pH. The relative enzyme activity was determined spectrophotometrically as per section 3.2.2 and 3.2.3.

3.4.4 Effect of different oils as carbon source on enzyme production

In order to determine the best carbon source for the fungus and lipid degrading capability on various vegetable oils 1% each of coconut oil, sunflower oil, mustard oil, soyabean oil was

added separately to 100 ml of the basal medium in place of olive oil. The contents were incubated at 28 ± 1°C and 5^{th} day culture broth of fungus was taken as crude enzyme extract and enzyme activity determined spectrophotometrically. Change in pH was observed in each media before and after cultivation i.e. after 5^{th} day.

3.4.5 Effect of different Organic nitrogen sources on lipase production

Both organic and inorganic nitrogen sources play an important role in enzyme synthesis. Inorganic nitrogen sources can be exhausted from the culture media quickly, while organic nitrogen sources can supply many cell growth factors and amino acids, which are needed for cell metabolism and enzyme synthesis.

Generally, microorganisms provide high yields of lipase when organic nitrogen sources are used. In present study lipid degrading capability was studied using different organic sources at 0.5% concentration. These were urea, casein, yeast extract and soyabean meal which were replaced with peptone in basal medium. Lipase activity was determined after 5^{th} day of incubation following same protocol (Section 3.2.2 & 3.2.3).

3.4.6 Effect of metal ions on the lipase activity

The effect of different divalent metal cation salts (both chloride anions and sulfate anions) and the chelating agent ethylenediamine tetraacetic acid (EDTA), on the lipase activity of the enriched lipase fraction was evaluated. The enriched lipase fraction (supernatant) was incubated for 30 min with Ca^{2+} ($CaCl_2$), Mn^{2+} ($MnCl_2$) as chlorides, Cu^{2+} ($CuSo_4$) or Zn^{2+} ($ZnSo_4$) as sulfates and EDTA all at 1mM concentration. The relative lipase activity was then evaluated taking the activity found in the control samples (without the addition of metal salts or EDTA) as 100%.

3.4.7 Statistical analysis

All experiments were performed in triplicates. The data were analyzed by analysis of variance and the means were compared with Tukey's test at $P < 0.05$. All the analyses were performed by using Graph Pad Prism 5.1 software and Costat software.

CHAPTER 4

RESULTS

4.1Qualitative screening of *Leptosphaerulina sp* for lipase production

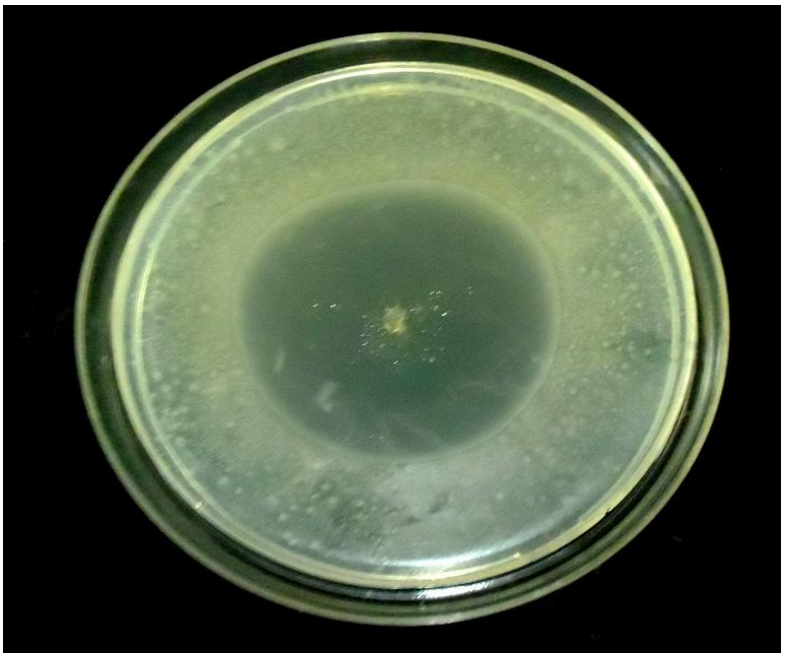


Fig 3: Tween 20 plate assay showing precipitation
around the fungus.

Tweens (fatty acid esters of polyoxyethylene sorbitan) have been the most widely used substrates for the detection of lipase producing microorganisms in agar media [Emanuilova *et al.* 1993]. Screening using tween agar plates showed precipitation around the fungus due to the precipitation as the calcium salt of the fatty acids which were released by hydrolysis of tweens. Liberated fatty acids bind with the calcium incorporated into the medium. The calcium complex is visible as insoluble crystals around the fungal disc (Fig 3).

4.2 Enzyme characterization

4.2.1 p-NP standard curve From table 1(Appendix 2)

Stock p-NP – 0.5 mM

Table 3: p-NP standard

Conc. p-NP (µM)	O.D 410nm
20	0.175
40	0.348
60	0.518
80	0.682
100	0.850
Blank	-

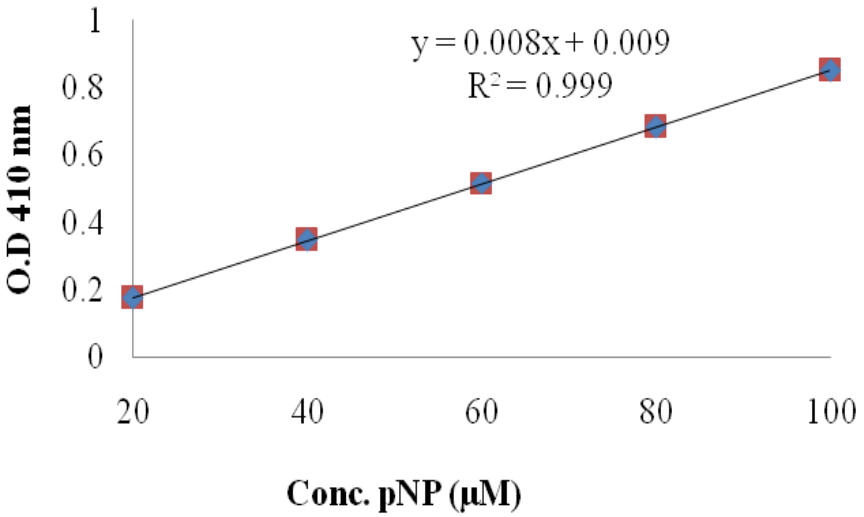


Fig4: Standard curve of p-NP

4.2.2 Assay of lipase activity-colorimetric assay

In order to check action of lipase on p-NPP initially, crude extract was taken after 5th day of incubation and assay was performed according to section 3.2.2. Lipase activity was calculated according to equation 1 & 2 (section 3.2.3).

After 30 minutes of incubation substrate i.e. p-NPP hydrolysed to release p-NP which gave yellow colour intensity of which was measured spectrophotometrically at 410 nm against enzyme free blank.

4.3 Bradford total protein assay

4.3.1 Standard curve of BSA

From table 2 (Appendix 2)

Table 4: Absorbance of BSA and protein sample

Conc. of BSA (µg/ml)	O.D 595 nm
20	0.373
40	0.626
60	0.987
80	1.413
100	1.863
Blank	-
Protein sample	0.563

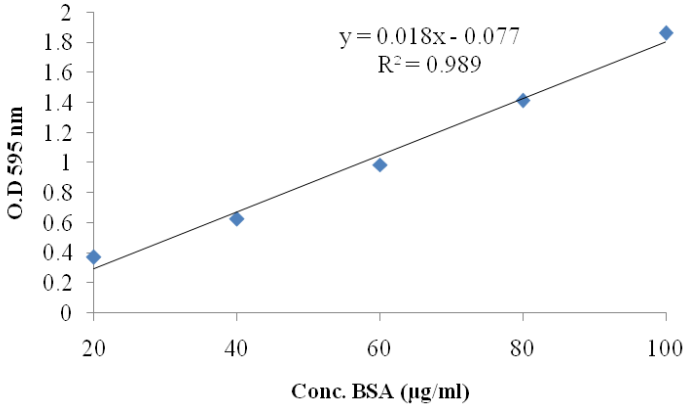


Fig 5: Standard curve of BSA

From straight line eq^{n}:

$$\text{Value of x (protein concentration)} = \frac{0.077 + \text{O.D (protein sample)}}{0.018}$$

= 35.5 µg/ml or 0.035 mg/ml

According to equation 1, 2 & 3 (section 3.2.3)

Table 5: Specific activity of lipase after 5 days of incubation

OD 410 nm	Conc p-NP (µM)	Specific Activity (U/mg)*
0.125	14.5	189.6 ± 0.1

***** Mean ± SD shown in table (n = 3)

Olive oil acts as an inducer in this case and mycelium growth was obtained by continuous shaking of culture. This indicates that organism requires large quantities of dissolved oxygen for their mycelia growth, multiplication and production of lipase.

4.4 Biochemical characterization of enzyme

4.4.1 Determination of Optimum Lipase Production Time

Table 6- Enzyme activity analysis table after different incubation period

Incubation time (days)	Specific Activity(U/mg) *	Final pH
2	107.2 ± 3.3 **d**	6.93 ± 0.03 **a**
4	153.2 ± 3.5 **b**	6.89 ± 0.04 **a**
6	173.7 ± 4.5 **a**	6.84 ± 0.04 **b**
8	153.9 ± 3.2 **b**	6.85 ± 0.04 **b**
10	128.7 ± 7.6 **c**	6.75 ± 0.03 **c**
LSD ($P < 0.05$)	0.225	

The values sharing a common letter within the column are not significant at $P < 0.05$

*Mean ± Std. deviation is shown in table (n = 3)

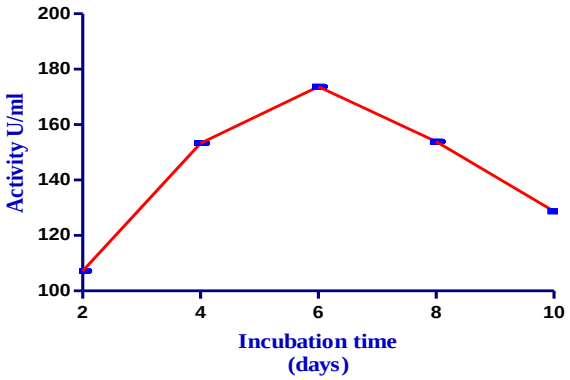


Fig 6: Effect of incubation period on lipase activity of *Leptosphaerulina sp*

The incubation time is an important factor for the production of extracellular lipase by the microorganisms [Shirazi *et al.* 1998]. The lipase activity was observed over a period of 10 days after two days interval each. The maximum lipase activity was observed on sixth day (Fig 6) but when compared with lipase activity on 5th day it was found to be little lower (Table 5). Hence we can say that maximum lipase production was observed after 5th day of incubation. At longer incubation periods, the lipase activity decreased which might be due to the depletion of nutrients, accumulation of toxic end products, and the change in pH of the medium, or loss of moisture. pH change observed in medium was not significant because long chain fatty acids have very poor solubility in aqueous solutions, hence affect pH rarely. Activity of lipase after 4th and 7th day was not significant according to table 6. Several researchers have reported different incubation periods for optimal lipase production. Maximum lipase activity was achieved after 48 h of incubation with *Rhizopus oryzae* by Ul-Haq *et al.* (2002). Cordova *et al.* (1998) reported the maximum lipase activity by *Rhizopus pullis* after 24 h of incubation using the mixture of olive oil cake and sugar cane bagasse as substrate. In another study, the maximum lipase activity by *Aspergillus niger* occurred after 5 days of incubation [Mahadik *et al*. 2002]. Benjamin and Pandey (1997) obtained maximum production of lipase by *Candida rugosa* after 3 days of incubation.

4.4.2 Effect of glucose as additional Carbon Source

After supplementing the media with glucose alone and in combination with olive oil activity of lipase was checked after 5 days of incubation using p-NPP assay (Section 3.2.2)

Table 7 – Enzyme Activity analysis table in presence of glucose as a sole carbon source and in combination with olive oil

Additional carbon source	Specific Activity (U/mg) *
Glucose	156.8 ± 2.8 **c**
Glucose + olive oil	175.6 ± 4.6 **b**
Olive oil	181.9 ± 8.2 **a**
LSD ($P < 0.05$)	0.257

*Mean ± Std. deviation is shown in table (n = 3)

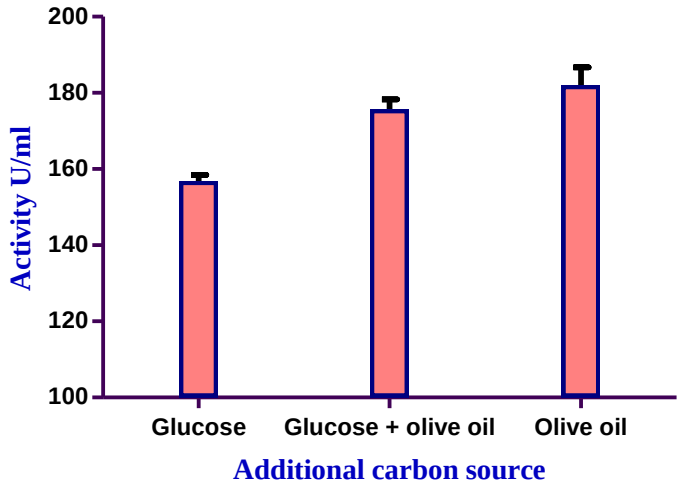


Fig 7: Effect of glucose/glucose + olive oil as additional carbon source on lipase activity of *Leptosphaerulina sp*

After 5 days of incubation lipase activity of 181.9 U/mg was observed in the medium containing 1% olive oil which was the basal medium (BM). The presence of 1% glucose, although stimulated lipase activity but it abolished the enzyme induction produced by 1% olive oil. The results presented in Table 7 and Fig 7 indicates a higher enzyme production in 1% olive oil medium in relation to glucose or glucose/olive oil medium (156.8 U/mg & 175.6 U/mg, respectively). However, the presence of both supplements depresses lipase induction. It is known that glucose inhibits the synthesis of most enzymes, and was not different for the lipases considered in this study. Glucose also repressed lipase production by *Candida rugosa* [Valero *et al.* 1991].

4.4.3 Effect of pH on lipase activity and stability

5th day enzyme extract which was showing maximum activity was taken and assay performed according to section 3.2.2 using different buffers of varying pH 4-12 (section 3.4.2.). The enzyme was shown to be reasonably active over a broad pH range of 8 to 12 (Fig 8) with a weaker activity level at pH 3 to 7, and with a pH optima of 11.0 (Relative activity at this pH was taken to be 100%). Supakdamrongkul *et al*. (2010) studied that lipase from *Nomuraea rileyi* showed pH optima of 9.0. Similarly *Rhizopus oryzae* lipase was found to be best active at pH 8.5 [Hiol *et al*. 2000].

Table 8- pH variation analysis table

pH	O.D 410nm	Relative activity %
4.0	0.014	3.6
5.0	0.020	4.8
6.0	0.049	10.2
7.0	0.196	34.4
8.0	0.309	58.0
9.0	0.336	62.3
10.0	0.435	88.4
11.0	0.480	100
12.0	0.325	60.0

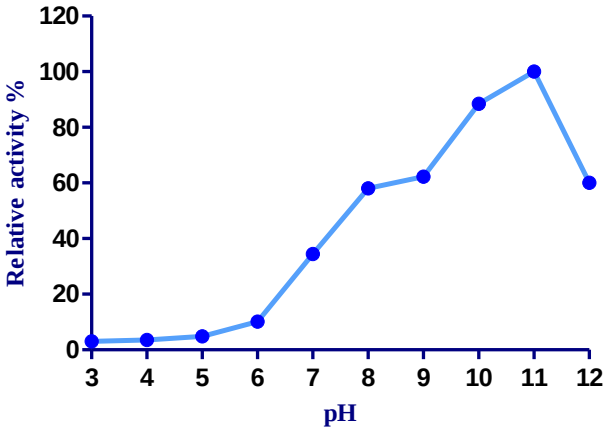


Fig 8: Graph showing pH stability of enzyme over pH 4-12

The lipase from *Leptosphaerulina sp* retained around 90% and 60% activity after incubation 30 min at pH 10 and 9 & 12, respectively (Table 8). The lipase exhibited pH and temperature kinetics that are potentially suitable for the detergent industry, as the enzyme is active at neutral to alkaline pH. Further characterization of the lipase was therefore carried out to evaluate it as a potential additive.

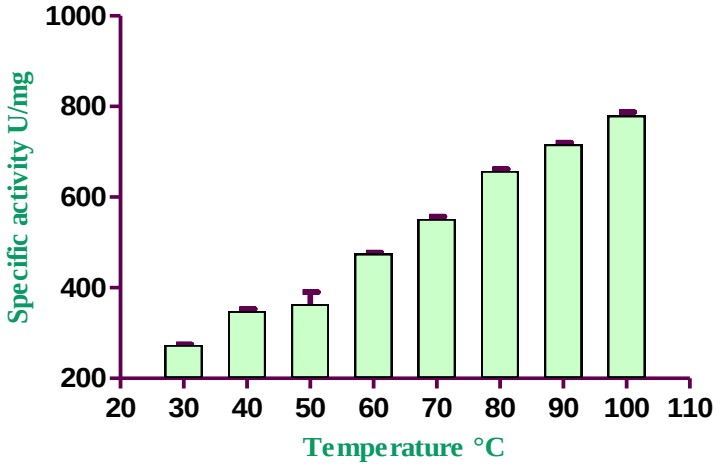
4.4.4 Effect of temperature on the activity and stability of lipase

Following were the results obtained after incubating enzyme and substrate solution over temperature gradient of 30-100°C (section 3.2.2). As shown in Fig 9, the optimal temperature of the lipase activity at pH 11 was 100°C. Below the optimal temperature, the activity decreased sharply with almost 10% with every 10 degree change in temperature. Lipase retained more than 50% stability at temperatures above 50°C. There was not a significant difference in activity of enzyme at 40°C and 50 °C. Even after 1 hour of incubation much change was not observed in lipase activity but activity started to decline after 1 hour of incubation.

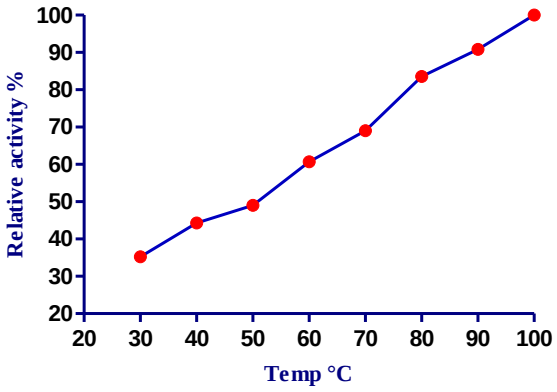
Table 9 – Temperature variation analysis table

Temperature °C	Specific activity (U/mg) *	Relative activity %
30	273.4 ± 3.2 **g**	35.2
40	348.3 ± 8.6 **f**	44.3
50	363.5 ± 46.3 **f**	49.0
60	475.1 ± 4.9 **e**	60.7
70	557.4 ± 9.2 **d**	69.0
80	657.3 ± 7.3 **c**	83.6
90	716.6 ± 5.9 **b**	90.8
100	779.9 ± 13.6 **a**	100
LSD (*P* < 0.05)	31.29	

*Mean ± Std. deviation is shown in table.
The values sharing a common letter within the column are not significant at $P < 0.05$



(A)



(B)

Fig 9: Graphs showing specific activity (A) and relative activity (B) of enzyme over temperature gradient 30-100°C

In this case CAPS buffer was used during incubation because unlike other buffers it does not lose its much activity at even 100°C. Highest specific activity was obtained at 100°C (785.7 U/mg) and lowest specific activity was 277.14 U/mg at 30°C (Table 8). Shangguan *et al.* 2011 reported that maximum temperature for *Aspergillus fumigatus* was 70°C. Till know no organism has been reported to show temperature optima of 100°C for lipase activity.

4.4.5 Effect of different oils used as carbon source on enzyme production

Table 10 – Lipase activity in presence of different edible oils and pH change of media after enzyme production.

Carbon sources	**Specific activity (U/mg) ***	**Final pH**
Mustard oil	884.8 ± 4.7 **b**	6.77 ± 0.04 **b**
Soyabean oil	940.1 ± 22.6 **a**	6.85 ± 0.03 **a**
Basal media	785.3 ± 4.7 **c**	6.89 ± 0.03 **a**
Coconut oil	687.1 ± 3.2 **d**	6.79 ± 0.02 **b**
Sunflower oil	917.1 ± 14.8 **ab**	6.81 ± 0.03 **b**
LSD ($P < 0.05$)	22.83	

*Mean ± Std. deviation is shown in table.

The values sharing a common letter within the column are not significant at $P < 0.05$

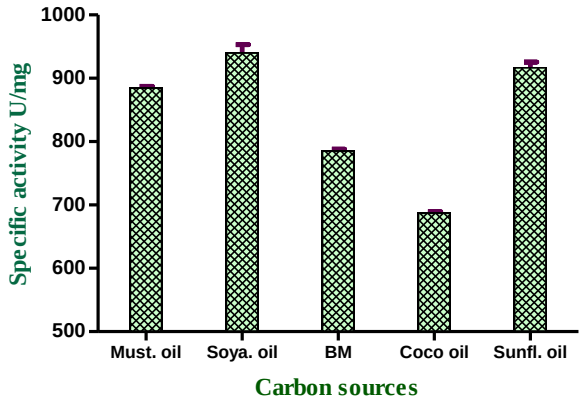


Fig 10: Graph showing specific activity of enzyme in presence of different oils in comparison to basal media.

As compared to basal media highest level of lipase was obtained when the fungus was grown in media supplemented with 1% soyabean oil (933.3 U/mg), which was followed by growth in sunflower (923.5 U/mg), and mustard oil (884.3 U/mg) (Table 10). Enzyme activity in presence of sunflower oil was not significant from mustard and soyabean oil. Soyabean oil may be considered an economically viable substrate for lipase production on an industrial scale. The regulatory mechanisms of lipase synthesis are different for each fungus, making this kind of study necessary. In some fungi, such as *Calvatia gigantean* [Christakopoulos *et al.* 1992] and *Rhodotorula glutinis* [Papaparaskevas *et al*.1992] the lipase production seems to be constitutive; but their levels were enhanced in the presence of oils. In others such as *Geotrichum candidum*, lipidic substrates are necessary for lipase production. [Shimada *et al*. 1992] The present study shows that oils are necessary for lipase production by *Leptosphaerulina sp*. These results indicate that the composition of vegetable seed oils (nature of the fatty acids constituting the acylglycerols) affect metabolism. Ul Haq (2002) investigated lipase production by *Rhizopus chinensis* and showed that soyabean oil enhanced lipase production and was highest among other oils studied (Fig 10). The highest levels were detected after 5 days of incubation at 100°C and pH 11.

4.4.6 Effect of different Organic nitrogen sources on lipase production

Table 11 - Lipase activity in presence of different organic nitrogen sources and pH change in each media**.**

Nitrogen sources	Specific activity (U/mg) *	Final pH
Urea	654.2 ± 11.9 **e**	8.85 ± 0.04 **a**
Yeast extract	878.9 ± 2.6 **b**	6.66 ± 0.03 **c**
Casein	776.5 ± 2.6 **d**	6.76 ± 0.03 **b**
Soyabean meal	985.4 ± 4.7 **a**	6.63 ± 0.04 **c**
Basal media	785.7 ± 3.3 **c**	6.80 ± 0.02 **b**
LSD ($P < 0.05$)	11.25	

*Mean ± Std. deviation is shown in table.
The values sharing a common letter within the column are not significant at $P < 0.05$

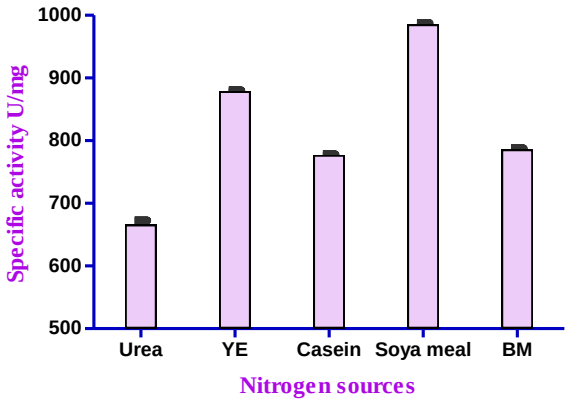


Fig 11: Specific activity of lipase in presence of different organic nitrogen sources in comparison with basal media

According to Table 11, highest production of lipase activity (985.4 U/mg) was found to be with 1% (w/v) soyabean meal (Fig 11) Hence it was the best organic nitrogen source. Yeast extract was also found to be an effective nitrogen source. To explain the superiority of peptone as an organic nitrogen source over other complex nitrogen sources such as casein, urea Freire *et al.* (1997) suggested that peptone contains certain co-factors and amino acids that match the physiological requirement for lipase biosynthesis. In case of urea pH of media was found to be the highest due to release of ammonia in the media.

4.4.7 Effect of different metal ions on the lipase activity

After incubation of enzyme extract with different metal ions (1mM) significant variation was seen in the activity of lipase.

Table 12 – Specific activity of lipase after incubation with different metal ions

Metal ions	Specific Activity (U/mg) *
$CaCl_2$	206.7 ± 6.1 **b**
$MnCl_2$	243.2 ± 6.9 **a**
$HgCl_2$	123.9 ± 4.9 **e**
$ZnSO_4$	146.4 ± 6.4 **d**
EDTA	181.4 ± 9.0 **c**
CONTROL	187.5 ± 4.6 **c**
LSD ($P < 0.05$)	11.63

*Mean ± Std. deviation is shown in table.
The values sharing a common letter within the column are not significant at $P < 0.05$

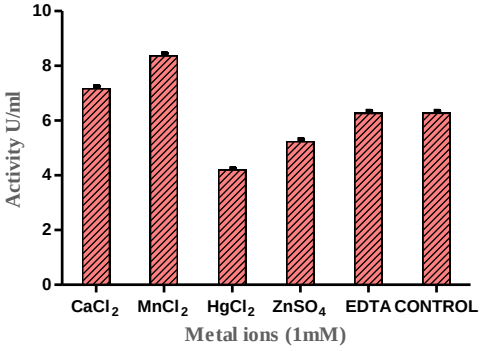


Fig 12 – Graph showing variation in activity of lipase due to different metal ions

The effect of the addition of 5 different divalent cation salts [three as chlorides and one as sulfate, and the chelating agent EDTA, at 1mM concentrations on the lipase activity is shown in Table 12. Ca^{2+} and Mn^{2+} ions stimulated the lipase activity, while Hg^{2+} and Zn^{2+} showed inhibition of lipase activity (Fig 12). Highest activity (243.2 U/mg) was obtained in presence of Mn^{2+} ions. It is consistent with that reported for the *Mucor sp* lipase [Abbas *et al.* 2002]. However in case of EDTA activity was not significantly different from control (no metal ions). Perhaps the Hg^{2+} & Zn^{2+} ions form a complex with the ionized fatty acids and change their solubility and behaviors at the oilwater interface. In addition, that Hg^{2+} inhibited the lipase activity could suggest the presence of at least one sulfhydryl group, most likely a cysteine amino acid residue, at the active site. Oxidation of this group by cations destabilizes the conformation folding of the enzyme, or leads to formation of disulfide bonds at irregular positions within the protein [Bera-Maillet *et al*. 2000].

CHAPTER 5

CONCLUSION

The levels of lipases produced by *Leptosphaerulina sp* were dependent on carbon source, and soybean oil was found to to be the best inducer. Glucose had a negative effect on this enzyme synthesis. The enzyme was found to be thermostable i.e. 100% stability was shown at 100°C and it can tolerate highle alkaline environment (optimum pH 11).

Due to the inducible, alkalophilic nature and stability of the enzyme, the *Leptosphaerulina sp* isolated from our present study can be exploited for the industrial production of alkalophilic lipase. The occurrence of an inducible, extracellular, alkalophilic lipase in fungi grown on vegetable oil is well documented in lipid biotechnology. A lipase that is stable at high alkaline conditions and high temperature is however rare, and in the present study we have isolated a fungal organism (*Leptosphaerulina sp*) which produces an inducible, extracellular, alkalophilic and thermostable lipase. There have been very few reports available so far with molds having alkalophilic and thermostable lipase (Licia *et al*., 2006; Ginalska *et al*., 2004). Therefore, the organism reported in this work can be exploited for commercialization as lipase of these characteristics finds immense application as additives for washing powders. However, further work is clearly needed to purify the enzyme and to study enzyme kinetics and also to increase the production of lipase as the activity is comparatively less than the strains used for commercial production.

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APPENDIX 1

1.1 Tween 20 Agar media

Peptone	1g
NaCl	0.5g
$CaCl_2.2H_2O$	0.01g
Agar	2g
Tween 20	1 ml

1.2 100 ml Basal medium

Peptone	0.5 g
$MgSO4.7H_2O$	0.05 g
KCl	0.05 g
KH_2PO4	0.2 g
$NaNO_3$	0.05 g
Olive oil	1ml

1.3 0.1M Potassium phosphate buffer (pH-7)

6.15 ml 1 M K_2HPO_4
3.85 ml 1M KH_2PO_4
Dilute to 100 ml using distilled water.

1.4 CAPS buffer (100 ml)

2mM CAPS – 44.2 mg
10 mM Na_2HPO_4 – 0.141 g
10mM Tris- HCl – 0.157 g

1.5 Preparation of Coomassie Reagent/Bradford reagent:

10.0 mg CBB G-250
5 ml of 95 % ethanol
10.0 ml of 85 % phosphoric acid
Final Vol. 100 ml with distilled water.

1.6 Buffers for pH optimization

0.1M Sodium Acetate (100 ml)

pH	0.1M Acetic acid	0.1M Sodium Acetate
3	98.23 ml	1.77 ml
4	84.7 ml	15.3 ml
5	35.7 ml	64.3 ml

0.1M Potassium Phosphate (Final vol.100 ml using Distilled water)

pH	1M KH_2PO_4	1 KH_2PO_4
6	1.32 ml	8.68 ml
7	6.15 ml	3.85 ml

0.1 M Tris Hcl (100 ml)

2.42 g Tris base + 1.5 ml of 1N HCl in 100 ml distilled water.

0.1 M Glycine – NaOH (100 ml)

Solution (a): Dissolve .750g of Glycine and .585g of Sodium Chloride in water and make up to 100ml

Solution (b): 0.1M Sodium Hydroxide

pH	Solution (a)ml	Solution (b)ml
9.0	8.8	1.2
10.0	6.0	4.0
11.0	5.1	4.9
12.0	4.5	5.5

APPENDIX 2

Table 1 – Preparation of p-NP standard

Stock- 0.5 mM

Conc. para-nitrophenol (µM)	Vol. from 500 µM stock (µl)	Vol. of buffer (ml)
20	120	2.88
40	240	2.76
60	360	2.64
80	480	2.52
100	600	2.4
Blank	-	3

Table 2 - Preparation of BSA standard

Stock: 1 mg/ml BSA

Conc. of BSA (µg/ml)	Vol. of BSA (1mg/ml)	Vol. of water (ml)	Bradford reagent vol. (ml)
20	40	1.96	3
40	80	1.92	3
60	120	1.88	3
80	160	1.84	3
100	200	1.8	3
Blank	-	2	3