Novel esterases from microbes through classical and metagenomics approach: Studies on the enzymes and their applications

Thesis submitted under the Faculty of Science of the
Cochin University of Science and Technology
For the award of the degree of

Doctor of Philosophy
in

Biotechnology

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March 2014

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DECLARATION

I hereby declare that the work presented in this thesis entitled "**Novel esterases from microbes through classical and metagenomics approach: Studies on the enzymes and their applications**" is a bona fide record of the research carried out by **Ms Deepthy Alex** (Reg No.3453), under my guidance and supervision, at the CSIR-National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram, India. I declare that all suggestions made by the audience during Pre-synopsis seminar and recommended by the doctoral committee have been incorporated in this thesis. I also declare that this work or no part of this has been submitted for the award of any degree, diploma, associateship or any other title or recognition.

Rajeev Kumar Sukumaran

Thiruvananthapuram
20 March 2014

DECLARATION

I hereby declare that the work presented in this thesis entitled "***Novel esterases from microbes through classical and metagenomics approach: Studies on the enzymes and their applications*****"** is based on the original work done by me under the guidance of Dr Rajeev Kumar Sukumaran, Senior Scientist, Biotechnology Division, CSIR-National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram, India and the thesis or no part of it has been submitted elsewhere for the award of any Degree, Diploma, Associateship or any other Title or Recognition.

Deepthy Alex

Dedicated to my Parents

Acknowledgements

Pursuing a PhD is indeed an adventurous experience accompanied with hardships, frustrations, encouragement and trust, a journey with so many kind and helpful hearts. As I reach the end of this journey, I realize that it was in fact, a team work that got me here. Words will not suffice to express my gratitude to all those who have helped me, yet I would like to leave a note of thanks to all who have been with me on this journey and helped me see it through.

First and foremost, I am deeply grateful for the constant support, insight and patience of my supervisor Dr Rajeev Kumar Sukumaran for his guidance without which this thesis would not have been possible. You have been a true mentor to me dear Sir. I would like to thank you for the faith you have had in me to accept me as your PhD student without any hesitation and also for all the constructive criticism and extensive discussions about my work thereafter.

I express my sincere gratitude to Dr Sarita G Bhat, Head, Department of Biotechnology, CUSAT and Expert Member of my Doctoral Committee for her valuable suggestions which helped in improving my research studies.

I would like to thank Prof Dr.Ashok Pandey, Head of Biotechnology Division, NIIST for his valuable suggestions and support throughout my research work. The regular quarterly reviews and seminars conducted in our division under his command has indeed groomed us a lot in developing our skills. I deeply appreciate your ways in managing our division.

I am thankful to Director, Dr. Suresh Das, Former Director, T.K Chandrasekhar, and Former acting Director Dr. B.C Pai, NIIST, CSIR, Trivandrum for providing me with all the facilities for completing my research.

My sincere and warm thanks to Dr.Sindhu for her valuable inputs and suggestions in my work especially in the metagenomic work. I extend my thanks to Dr. Madhavan Nampoothiri, Dr. Binod Parameswaran, Dr Ramesh, Dr. M Arumugham, Mr. Kiran Kumar and Dr. D Leena for their critical suggestions and help during my research work.

I take this opportunity to thank the Doctoral Committee and Research Committee of CUSAT, Academic Program Committee of NIIST and NIIST-CUSAT Research Council for all the timely help during the entire course of my work and thesis submission. I also thank all the administrative and supporting staff of CUSAT and NIIST for their support and help.

I am grateful to Dr. Vijaya Lakshmi Amma, Mr Sivan Kutty Nair, Mr. KM Prakash, Ms Radhamani, Ms Sasikala and Ms Rajalakshmi for their assistance in my work. I also acknowledge the supporting staff in NIIST especially the Library and Administration staff.

Team Biotech has been a wonderful community in which to 'grow up' as a research student: the personal and professional relationships forged in these years will stay with me forever. I am particularly grateful to Vidya, Ushasree, Arya and Neetha; my lab mates as well as roommates for

they have made my stay comfortable and memorable here. I deeply cherish the fun we had together for the past five years.

I extend my thanks to Rajasree and Abraham for all their generous and timely help. I express my gratitude towards my seniors Dr. Reeta, Dr Nisha, Dr Raji, Dr Sayed, Dr Aswathy, Dr Swetha, Dr Dhanya, Dr Gincy, Dr Sai and Dr Shyam who kept me going in the beginning. With immense pleasure I wish to extend my sincere thanks to my friends Vipin, Kiran, Aravind, Anusree, Deepa, Kuttiraja, Preeti, Divya, Varsha, Nimisha, Sabeela, Vani, Sajna, Arjun, Lalitha, Leya, Nishant, Soya, Ayman, Meena, Dilna, Vini, Sneha, Surya and all friends of my Division for their support, encouragement and care.

My sincere thanks to students of other divisions especially Janu, Linsha, the photochemistry section of CSIR-NIIST for providing me with DLS and AFM facilities and the AP & NP division for FAME analyses.

Words cannot express my deepest thankfulness to my parents for the support, concern, prayers and sacrifices they have offered without which it would not have been possible to reach where I am today. I am dedicating my thesis to my parents. Care and support of my brother Deepu and sister in law Elizabeth and my sister Deepa and brother- in- law Bino and their two wonderful kids Diya and Isa. I thank them all for the unconditional love they have showered on me.

Above all, I express my gratefulness to the Almighty for guiding me and strengthening me through all the difficulties and for making my paths straight, for I believe it's Your plans and not mine that has taken me through this wonderful journey.

Deepthy Alex

LIST OF PUBLICATIONS

Published in SCI journals

1. **Deepthy Alex**, Ashok Pandey, Rajeev K Sukumaran, 2014, Esterase active in polar organic solvents from the yeast *Pseudozyma* sp NII 08165, Enzyme Res (In Press) http://www.hindawi.com/journals/er/aip/494682/

2. Jajaja Vidya, Sweta Swaroop, Sudheer K Singh, **Deepthy Alex**, Rajeev K Sukumaran, Ashok Pandey, 2011, Isolation and characterization of a novel α-amylase from a metagenomic library of Western Ghats of Kerala, India, Jalaja Vidya, Sweta Swaroop, Biologia 66 (6): 939—944

Communicated

1. **Deepthy A**lex, Abraham Mathew, Rajeev K Sukumaran. 2014, Esterases immobilized on aminosilane modified magnetic nanoparticles as a catalyst for biotransformation reactions (Communicated to Bioresource Technology- Ref BITE-S-14-02007).

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1. Rajeev K Sukumaran, Lalith Devi Gottumukkala, Rajasree Kuniparambil, **Deepthy Alex**, Ashok Pandey, 2011, Butanol from Biomass: Revisiting ABE fermentation, In : Pandey A, Larroche C, Ricke SC, Dussap CG, Gnansounou E (eds) Biofuels: Alternative Feedstocks and Conversion Processes, Academic Press, London, pp-571-586

Papers/Poster Presented in Conferences/Seminars

1. **Deepthy Alex**, Ashok Pandey, Rajeev K Sukumaran, 2011, Purification and characterization of thermo and halotolerant esterase sfrom a metagenomic library constructed from soap factory effluent, In International Conference on "New Horizons in Biotechnology" organized by CSIR-NIIST and BRSI, Trivandrum Nov 21-24.

2. **Deepthy Alex**, Sindhu Raveendran, Ashok Pandey, Rajeev K Sukumaran, 2010, Esterase from soil DNA through metagenomics, In International conference on Genomic Sciences, Madurai Kamaraj University, Madurai, November 12 -14, 2010,

3. **Deepthy Alex**, Anju Shainu, Ashok Pandey, Rajeev K Sukumaran, 2009, Studies on temperature and solvent tolerant esterase from an yeast like isolate NII 08165. International Conference on Emerging Trends in Biotechnology and VI BRSI Convention, Banaras Hindu University, Varanasi, India, December 4-6

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Chapter 1
Introduction and Review of Literature

1.1. Introduction

Esterases comprise a diverse group of hydrolases that catalyze the cleavage and formation of ester bonds (Lopes *et al.,* 2011). Two major classes of hydrolases are of paramount importance: 'true' esterases (EC 3.1.1.1, carboxyl ester hydrolases) and lipases (EC 3.1.1.3, triacylglycerol hydrolases) (Bornscheuer, 2002). While carboxyl esterases preferentially hydrolyze water soluble esters and triacylglycerols with fatty acid chain lengths shorter than C6, lipases prefer water insoluble substrates, typically triacylglycerols with medium to long chain fatty acids (≥10 carbon atoms).

Lipases belong to the class of serine hydrolases and do not require any cofactor (Jingnan, *et al.,* 2013). Under natural conditions, they catalyze the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase in which the enzyme is dissolved. In the absence of water, they are capable of reversing the reaction. The reverse reaction leads to esterification and formation of glycerides from fatty acids and glycerol. The common industrial lipases are special classes of esterases that act on fats and oils, and hydrolyze them initially into the substituted glycerides and fatty acids, and finally on total hydrolysis into glycerol and fatty acids. One characteristic feature of lipases is the interfacial activation and the presence of a lid domain in the active site of the enzyme.

Lipases/esterases find applications in a variety of biotechnological fields such as food and dairy (cheese ripening, flavor development), detergent, pharmaceutical (naproxen, ibuprofen), agrochemical (insecticide, pesticide) and oleochemical (fat and oil hydrolysis, biosurfactant synthesis, structured lipids) industries (Pandey *et al.,* 1999; Saxena *et al.,* 1999; Alain *et al.,* 2004; Hasan *et al.,* 2006; Fazary and Ju 2008; Long *et al.,* 2011; Zhao *et al.,* 2014). Lipases may be further exploited in many novel areas where they might serve as potential biocatalysts.

Lipases and esterases catalyze reactions like esterifications, transesterifications and inter-esterifications and both show increased activity in oil water interphase, a property called interfacial activation (Martinelle *et al.,* 1995; Long *et al.,* 2011).

Lipases are widely found throughout the animal and plant kingdoms, as well as in molds and bacteria (Hamosh and Burns, 1977; Fink *et al.*, 1984; Villeneuve, 2003; Sammour, 2005). Among these, microbial lipases are largely exploited for industrial applications. Microbes can be easily cultivated and they offer a broad spectrum of lipases which can catalyze a wide variety of hydrolytic and synthetic reactions (Jaeger and Reetz, 1998; Benjamin and Pandey, 1998; Bradoo *et al.*, 1999; Ferreira-Dias *et al.*, 1999; Jaeger *et al.*, 1999 ; Gao *et al.*, 2000; Rathi *et al.*, 2002; Dimitrijevic, 2011). The synthesis and secretion of lipase by microbes are dependent on several parameters and quite often modulations in lipase production by the microbes may be brought about by modulations of external environment including culture media.

Lipases with novel properties are always in demand due to the immense number of synthetic reactions for which enzymatic routes are currently not available (Saxena *et al.*, 1999) Novel specificities, Tolerance to extremes of pH, temperature and/or salt tolerance are features often desired in lipases. Since majority of the industrial applications of lipases are in synthesis of compounds and in esterifications, the stability of enzyme in organic solvents and its ability to act in presence of elevated concentrations of solvent is highly desired.

Though the classical approach of screening a wide variety of microbes has yielded several novel lipolytic activities, this method does not fully exploit the microbial biodiversity since a vast majority of the microorganisms are difficult to be isolated and maintained in pure culture (Handelsman, 2004). Since a 'metagenomics' approach can help to discover genes for lipases from un-cultivable microbes, the possibility of obtaining novel activities are large. In this present study we tried to explore novel lipases through classical as well as metagenomic approaches. Studies on the desirable properties of the enzyme and possible applications have also been conducted.

1.2. Review of Literature

Lipases are triacylglycerol acyl hydrolases (E.C.3.1.1.3) that catalyze both the hydrolysis and synthesis of long chain triacylglycerols (Fernandes *et al.*, 2004). Lipases stand amongst the most important biocatalysts carrying out novel reactions in both aqueous and non aqueous media primarily due to their ability to utilize a wide spectrum of substrates, and stability towards extremes of temperature and pH. Determination of three-dimensional structures of lipases has thrown light into their unique structure–function relationship. Among lipases of plant, both animal and microbial origin, it is the microbial lipases that find important industrial applications (Verma *et al.*, 2012). The advantages of microbial lipases for industrial applications are that the microbes can be easily cultivated and their lipases can catalyze a wide variety of hydrolytic and synthetic reactions (Saxena *et al.*, 1999). Microbial lipases are comparatively more stable compared to their counterparts from animal or plant kingdom and can be obtained in bulk quantities at low cost.

1.2.1. Different reactions catalyzed by lipases

Lipases catalyze wide range of reactions, including hydrolysis, inter-esterification, alcholysis, acidolysis, esterification and aminolysis. They catalyze the hydrolysis of fatty acid ester bond in the triacylglycerol (TAG) and release free fatty acids (Harikrishna and Karanth 2002). Two basic reactions catalyzed by lipases include hydrolysis and esterification. The reactions catalyzed by lipases mainly depend on the presence of water. In low water medium they catalyze esterification, inter-esterification and trans-esterification reactions. Hydrolysis reactions are basically fat hydrolysis in presence of water and under mild conditions of temperature and pressure (Chua *et al.*, 2012). The products of hydrolysis reactions are fatty acids and di- and/or mono glycerides. Esterification reactions are opposite to that of hydrolysis reactions and here the enzyme catalyzes the formation of an ester bond. Mild reaction conditions, reduced side reactions and specificity are the important factors which make lipases- efficient systems for use in the synthesis of esters. Numerous high value chemicals are produced by esterification using lipases (Syamsul *et al.*, 2010). Trans-esterification on the other hand is the exchange of acyl radicals between an ester and an acid (acidolysis) or another ester (inter-esterification), or between an ester and an alcohol (alcohololysis) (Srinivas *et al.*, 2003)

1.2.2. Lipases and Esterases

Both esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) are the enzymes catalyzing the hydrolysis of ester bonds and are widely distributed in animals, plants and microorganisms. In organic media, they catalyze reactions such as esterifications, inter-esterifications or trans-esterifications. Both prefer an oil water interface for their activities. Also both the classes of enzymes have some conserved sequences which are important for maintaining their structural scaffold or to maintain specificity and activity. Lipases can be distinguished from esterases by the phenomenon of interfacial activation and/or by having a hydrophobic domain (lid) covering the active site of the enzyme (Lopes *et al.,* 2011). Besides, esterases are defined as enzymes that hydrolyze the triglycerides with short chains and prefer water-soluble substrates, while lipases are enzymes that hydrolyze triglycerides with long chains and prefer water-insoluble substrates. Lipases posses the unique feature of acting at the aqueous and non–aqueous interface which distinguishes them from esterases (Rahman *et al.,* 2012).

1.2.3 Sources of lipase

Lipases are ubiquitous enzymes with considerable physiological significance and industrial potential. The enzymes may be sourced from animals, plants or microbes though the latter is the most commonly exploited source for industrially important lipases. Mammalian lipases are classified in to four groups i.e, the pancreatic, gastric, hepatic and lingual lipases based on their locations (Turki and Kallel 2012). Pancreatic lipases are secreted in to duodenum and act on dietary triglycerols hydrolyzing them in to free fatty acids and glycerol. All gastric lipases are active and stable under acidic conditions. Gastric lipases are secreted by cells located in the fundic region of stomach are presumed to initiate the digestion of triacyl glycerols. Lingual lipases secreted by serous glands of tongue catalyze hydrolysis of triacyl glycerols in to fatty acids and di or mono acyl glycerols under acidic environment in the stomach (Newsholme and Leec, 2010). Hepatic lipases are lipolytic enzymes made by the hepatocytes in liver. Plant lipases are attractive due to their low cost and easiness of purification. Lipase sourced from Papaya latex is used in oil and fat modifications (Villeneuve, 2003).

1.2.3.1 Microbial lipases

Lipases widely occur in bacteria (Casas-Godoy *et al.,* 2012; Thaku, 2012) yeasts (Rapp and Backhaus 1992; Dalmau *et al.,* 2000; Vakhlu and Kour 2006) and fungi (Singh and Mukhopadhyay, 2012). Among microbial lipases, lipases of bacterial and fungal origin are most studied and also commercially exploited.

Extra-cellular lipase production by bacterial strains is of immense importance because of the ease of their production. Although there are number of bacterial strains which produce lipases, only few of them have been commercially exploited (Rajendran *et al.,* 2009; Palekar *et al.,* 2000). Lipases from genus *Pseudomonas* are widely used for biotechnological applications (Beisson, et al. 2000). *Pseudomonas cepacia, Pseudomonas aeruginosa, Pseuudomonas fluorescens, Pseudomonas fragi, Bacillus thermocatenulatus, Staphylococcus hyicus, Staphylococcus aereus, Staphylococcus epidermidis* are some of the bacterial species producing lipases (Jaeger adn Reetz, 1998; Reetz *et al.,* 1998; Rua *et al.,* 1998; Simons *et al.,* 1998; Rajendran *et al.,* 2009)

Yeast lipases are generally extracellular, monomeric glycoproteins although some lipases from *Yerrowia lipolytica* are reported to be intracellular (Merek, 1996; Pignede *et al.,* 2000). *Candida antarctica, C. curvata, C.cylindracea, C.parapsilosis, C.rugosa, C. tropicalis, Pichia bispora, Saccharomyces lipolytica, S .crataegenesis, Torulospora globora,* and *Yarrowia lipolytica* are some of known yeast species producing lipases (Jaeger and Reetz, 1998; Arroyo, 1999)

Among the filamentous fungi, Mucorales have been studied in great details which include lipases from *Mucor hiemalis, Mucor miehei, Mucor pusilus, Rhizopus japonicus, Rhizopus arrhizus, Rhoizopus delimar, Rhizopus nigricans (Lazar and Schroder 1992), Rhizomucor meihei, Pencillium camberti, Humicola lanuginosa, Rhizopus oryzae, Aspergillus niger* are some other fungal strains producing lipases (Merek, 1996; Dellamora-Ortiz *et al.,* 1997; Jaeger and Reetz, 1998)

1.2.4. Specificity of Lipases

Lipases are amongst the most important biocatalysts carrying out novel reactions in both aqueous and non aqueous media. This is primarily due to their ability to utilize a wide spectrum of substrates, high stability towards extremes of temperature, pH and

organic solvents, and chemo-, regio- and enantio selectivity. In nature, the lipases available from various sources have considerable variation in their reaction specificities. This property is generally referred as enzyme specificity (Long, 2010). The specific reaction catalyzed by lipase for a given substrate under given lipolytic conditions can be said to be its finger print. Thus some lipases have affinity for short-chain fatty acids - acetic, butyric, capric, caproic, caprylic, etc (Saxena *et al.,* 1999) while some have preference for unsaturated fatty acids-oleic, linoleic, linolenic, etc many others are nonspecific and randomly split the fatty acids from the triglycerides. Lipases often show positional specificity and attack the fatty acids at 1 or 3 carbon positions of glycerol or at both the positions but not the fatty acid at the 2nd position of the glycerol molecule (Saxena *et al.,* 1999). However, through random acyl migration, the 2-fatty acid mono glyceride undergoes rearrangement pushing the fatty acid to the 1 or 3 position of the glycerol molecule; as acyl migration is a slow process and as the available lipases do not act on glycerol 2-mono fatty acid esters, the hydrolysis slows down and unless acyl migration is complete, the lipases cannot attack the fatty acid at sn2 position. Till date, there are no authentic reports of lipases which catalyze the release of fatty acids selectively from the central 2-position of acyl glycerols, except for a report from Asahara *et al.,* (1993). Lipases are regio-specific and stereo-specific. Stereo-specificity of lipases is the ability to differentiate between two enantiomers in a racemic mixture and regio specificity is defined as the ability of lipases to distinguish between *sn*-1 and the *sn*-3 position of TAG. The regio selectivity for the *sn*-3 and *sn*-1 positions by lipases has been widely used in the resolution of racemic mixtures of esters other than acyl glycerol. Partial stereo specificity in the hydrolysis of triacyl glycerols has been observed in *Rhizopus arrhizus, R. delemar, Candida. cylindracea,* and *Pseusomonas aeruginosa* (Asahara *et al.,* 1993). Owing to this property, these enzymes can be used to isolate optically pure esters and alcohols. Interestingly, lipases function at the oil–water interface. The amount of oil available at the interface determines the activity of the lipases. This interface area can be increased substantially to its saturation limit by the use of emulsifier as well as by agitation (Saxena *et al.,* 1999), The saturation limit depends on the ingredients used as well as the physical conditions deployed. Thus, the activities of lipases can be pronouncedly increased by use of emulsifying agents as well as by methods that increase the size of the emulsion micelles.

1.2.5. Active site of lipase and the mechanism of action

The 3D structure of lipase was first elucidated in human pancreatic lipase (Winkler *et al.*, 1990) and *Rhizomucor miehei* lipase (Brady *et al.*, 1990). Lipases share a α/β-hydrolase fold with a central, mostly parallel, β-sheet and the mechanism of hydrolysis is based on the catalytic triad as observed in serine hydrolases (Winkler and Gubernator 1994). The catalytic triad of lipases is composed of three amino acids -Serine, Histidine and Aspartate/Glutamate, which are far apart in the primary sequence but spatially very close in the folded protein (Brumlik and Buckley 1996). The active site of lipases in the 'closed' form is shielded from the surface by protective surface loops called the 'lid'. Upon activation, the lid undergoes a conformational rearrangement exposing the active site serine and creating the active, open form of the enzyme facing toward hydrophobic side (Brzozowski *et al.*, 1991; Nardini *et al.*, 2000).

The presence of catalytic triad in the active site of the enzyme similar to that in serine proteases indicates that both has similar catalytic mechanisms (Winkler and Gubernator, 1994). There are two elementary steps involved in hydrolysis which are

i. The formation of non covalent Michaelis complex
ii. Nucleophilic attack by the catalytic site serine-oxygen on a carbonyl carbon atom of ester bond, leading to formation of a transient tetrahedral intermediate stabilized by hydrogen bonding with two peptide amino groups (Cygler *et al.*, 1997).

The lipase activity is found to be maximum at an oil -water interface (Thuren, 1992). The studies done in *Candida rugosa* suggests that the active state of the enzyme is not its final state and it is the intermediate state .The highest reaction profile was at higher pressures in the interface indicating that the activation of the enzyme is surface pressure dependent (Rao and Damodaran 2000).

1.2.6. Lipases with important properties

1.2.6.1. Solvent tolerant lipases

Triacyl glycerols with short chain fatty acids tend to dissolve in water while those with larger chain length are water immiscible (Klibanov and Alexander, 2001). This causes problems in studying the properties of the enzyme. Use of organic solvents has great advantage over using water which includes the relatively higher solubility of substrates, the relative ease of recovery of products in organic phase, the possibility of reducing the degree of undesirable substrate and/or product inhibition in organic solvent-water two-phase system and the ability to shift the reaction equilibrium in the synthetic direction when the products are continuously removed, in the case of an organic solvent water-two phase system (Hun *et al.,* 2003). Solvent tolerant lipases are also desirable in applications were the desired reaction will take place only in presence of a solvent.

1.2.6.2. Thermostable lipases

Thermostable lipases are obtained largely from *Bacillus* and *Pseudomonas* species. Thermostable lipases are important due to their applications in reactions which might need elevated temperatures. Also some fats assume liquid form only when they are at higher temperatures and their hydrolysis can proceed only at liquid state. Thermostable lipases can act at elevated temperatures and complete such reactions (Sugihara *et al.,* 1991; Gao *et al.,* 2000)

1.2.6.3. Lipases showing tolerance to NaCl (Halo tolerant lipases)

Salt tolerant lipases are desirable in several industrial applications including food industry (Bruni *et al.,* 1982). While marine microbes are a natural choice as source of salt enzymes, most of the enzyme sourced from such organisms may not act in the absence of salt. Enzymes from mesophiles with a broad range of NaCl tolerance are often desired for majority of the applications due to the broad range of tolerance. (Rathi *et al.,* 2001). While there are several producers of NaCl tolerant lipases, major sources of such enzyme are species from *Pseudomonas* species. The microbes producing enzymes with a broad range of NaCl (0-3M) tolerance –are described by Amoozegar *et al.,* (2008).

1.2.7. Applications of lipases

The last quarter of the 20th century has witnessed unprecedented use of lipases in biotechnology, manufacture of pharmaceuticals and pesticides, single cell protein production, biosensor preparation and in waste management etc (Torossian and Bell, 1991; Gandhi, 1997; Yadav, 1997; Jaeger and Reetz 1998; Pandey *et al.,* 1999; Saxena *et al.,* 1999; Hasan *et al.,* 2006; Amoozegar *et al.,* 2008). Lipases have found applications in chemical synthesis as well as in medicine, industrial chemical processes etc. Lipase-supported synthesis of biologically active compounds and pharmaceuticals, use of preparative biotransformations in organic chemistry, lipase-catalyzed ester synthesis, use of lipases in racemic resolutions and polymer synthesis and several other applications have been developed and perfected in past several years (Houde *et al.,* 2004). Limitations of the industrial use of these enzymes have mainly been owing to their high production costs, which may be overcome by molecular technologies, enabling the production of these enzymes at high levels and in a virtually purified form (Saxena *et al.,* 1999).

Lipases are used extensively in the dairy industry for the hydrolysis of milk fat which is necessary in several applications like flavor enhancement of cheese, acceleration of cheese ripening, manufacture of cheese-like products, and lipolysis of butter fat and cream (Falch, 1991). Moreover, the addition of lipases cause the production of free fatty acids which take part in simple chemical reactions where they initiate the synthesis of other flavor ingredients such as aceto-acetate, ß-keto acids, methyl ketones, flavour esters, and lactones (Kinsella and Hwang, 1976)

Another major use of lipases is as additives in detergents. The usage of enzymes in washing powders still remains the single biggest market for industrial enzymes (Jaeger and Reetz, 1998).The world-wide trend towards lower laundering temperatures has led to much higher demand for household detergent formulations. Lipases used as detergents include those from *Candida* (Nishioka and Takama, 1990), *Chromobacterium* (Minoguchi and Muneyuki, 1989). Commercial lipases used as detergents include those from *Pseudomonas psuedoalkaligens* and *from Pseudomonas mendocina*.

The scope for application of lipases in the oleochemical industry is enormous. The introduction of the new generation of cheap and highly thermo-stable enzymes can change the economic balance from chemical synthesis in favor of lipase use for several compounds. The various reactions involving hydrolysis, alcoholysis, and glycerolysis

have been carried out directly on mixed substrates, using a range of immobilized lipases (Hoq, 1985; Arbige, 1989; Buhler, 1987). Enzymatic hydrolysis perhaps offers the greatest hope to successful fat splitting without substantial investment in expensive equipment as well as in expenditure of large amounts of thermal energy. The major applications in the oleochemical industry include synthesis of di-glycerides (Ergan *et al.,* 1988), surfactants (Adelhorst *et al.,* 1990), additives for cosmetics and personal care products (Alain *et al.,* 2004), pharmaceuticals, agrochemicals in the manufacture of polymers and in the production biodiesel (Noureddini *et al.,* 2005), leather (Muthukumaran and Dhar, 1983) and waste water management (Knezevic *et al.,* 1995; Lin and Lo, 1997)

1.2.8. Fermentative production of lipases and optimization of production

Although lipases have been used for several years to modify the structure and composition of fats and oils, they only recently became available for large-scale use in industry, due to the high enzyme cost and appropriate selectivity for industrial purposes. In this regard, industry continues to look for economical sources of lipases with high activity and characteristic selectivity. The major constraint in the use of lipases for large scale applications is the cost of the enzyme. A significant part of this cost is contributed by the expense of production, and any improvement in the production economics is greatly welcome by the industry. Majority of the natural sources of lipases produce the enzyme at lower titres and the production of lipases often has to be improved for commercial scale exploitation. Lot of research has been directed at the large scale cultivation of *Candida rugosa* the major source of industrial lipases. The main focus of the research has been founded on the submerged fermentation technology (Benjamin and Pandey, 1996). In majority of the works on *C. rugosa* lipase production, olive oil or some other lipid was added as the carbon source (Velero *et al.,* 1991). Solid state Fermentation technology was also tried successfully in several instances for CRL production (Benjamin and Pandey 1997, 1998). Both SmF and SSF technologies have been employed for lipase production from other microorganisms also (eg Kamini *et al.,* 1998, Gombart *et al.,* 1999, Elibol and Ozer, 2002, Tan *et al.,* 2004) with varying amounts of success. While majority of these studies dealt with empirical optimization of process

parameters for improving lipase production there also has been statistical approaches in experiment design and analysis of lipase production (Elibol and Ozer 2002)

Lipase production can be increased by strain improvement and optimization of fermentation conditions. Media composition, physiochemical and cultural factors of the microbes play a vital role in the efficiency and the economics of the entire process. In the conventional method for the optimization of enzyme production, the “one variable at a time” approach is used, which involves changing one parameter at a time while keeping the other entire parameters constant (Greasham and Inamine, 1986; Duan *et al.*, 1994). The optimized concentration of the variable factor of the previous experiment is then incorporated in the next experiment .The same procedure is followed for all the parameters to complete the optimization (Young and Bratzler, 1990). But this process is cost, labor and time intensive, and also does not consider the interaction between different variables. An alternative and more efficient approach is the use of statistical methods. Several statistical methods ranging from two factorial to multi factorial designs are available (Monagan and Koupal, 1989).

Plackett & Burman design and Response Surface Methodology are employed for identifying the important parameters in the fermentation process and optimizing these for obtaining maximum enzyme production. PB design is a two level screening design (Plackett and Burman, 1946) which is used to screen the effect of the parameters on product formation Maximum number of variables that can be handled is N – 1 ,where N is the number of variables. Two values for each variable, ie - a high (+ 1) and a low (- 1) value is set, such that the difference between the two values should be large enough to ensure that the peak area for maximum enzyme production is included. The design matrix for PB designs can be obtained using software packages for design of Experiments (DOE) like Design Expert (Statease Corp, USA) and Statistica (Statsoft Inc, USA). Each factor is tested for an equal number of times at its low and high settings. A statistical analysis is performed thereafter to find the impact of each parameter on the process. The effect of parameters is estimated as the difference between the mean responses at high and low levels of each variable. The magnitude determines the importance of the parameters and the direction (+ or -) gives the information on whether the effect was positive or negative. Significant variables are then identified using a regression analysis.

In majority of the cases, further optimization of the significant variables identified by a factorial design like Placket and Burman design are done using response surface methodology (RSM). All the non –critical factors are fixed at a particular level mostly that which has produced the maximum response in the screening experiment or sometimes at their median values. The levels of parameters are three in this case and includes a middle level (0) an two equally spaced levels , one higher (+1) and the other lower (-1). The RSM evaluates the quadratic effects and interactions amongst the variables. The main objectives of the optimization study are to find the interaction effects and to find the optimal settings of the parameters. The Box-Behnken Design (Box and Behnken 1960) is a type of optimization design that has been widely used in the optimization of enzyme production. The response can be found out from the polynomial equation;

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_{23} + \beta_{23} X_2 X_3 + \in$$

Where

y	- Response
x_1, x_2, x_3	- Coded settings
x_1^2, x_2^2, x_3^2	- Quadratic effects
$x_1 x_2, x_2 x_3, x_1 x_3$	- Interactions

The interaction of one variable with that of the other can be determined by three dimensional response surface plots or contour plots (3- D plots). The optimum concentrations can be made derived by solving the polynomial model equation, which is often validated experimentally.

1.2.9. Screening for lipases

Screening methods are required to identify or select a specific enzyme producing strain from a group of organism or clones. Function based screening as well as sequence homology based screening methods are the two different approaches conventionally used in screening of lipases. Sequence based screening approach by PCR using primers designed based on the consensus sequence of a catalytic triad and oxyanion hole of lipases gained attraction in the last decade (Bell *et al.*, 2002; Hyung *et al.*, 2004).

Functional screens on the contrary make possible the selection of enzymes based on activity. Moreover it enables the identification of novel enzymes that exhibits no sequence similarity with known enzymes especially while screening metagenomic libraries. Tributyrin agar emulsified with gum arabic and egg yolk plates are conventionally used for esterase/lipase screening (Mourney and Kilbertus, 1976; Kok *et al.,* 1993). Esterase production can be detected by zone of clearance around the microbial colonies. A more sensitive and specific plate assay for lipases in medium containing trioleoyl glycerate and fluorescent dye rhodamine was reported by Kouker and Jaeger (1987). Lipase producing organisms form orange fluorescent halos around colonies which can be visible upon UV irradiation. A successful plate screening method using Tween 20 was reported by Salihu *et al.,* (2011). Function based novel screening methods for esterases, lipases and phospholipases to explore the uncultivable microorganisms was described by Reyes-Duarte *et al.,* (2012). High throughput screening methods for lipases using synthetic chromogenic substrates (Mateos-Díaz *et al.,* 2012) and novel methods with GC based multi substrate kinetic resolution are the advancing areas for screening lipases (Osze *et al.,* 2012).

1.2.10. Purification of lipases

Most of the purification steps attempted to date includes a precipitation/extraction step followed by a combination of chromatographic techniques. Usually precipitation is done with ammonium sulfate / organic solvents like ethanol or acetone. Enzyme loss due to denaturation of protein is one of the disadvantages while concentrating lipase with organic solvents. A single chromatographic technique may not be enough to purify an enzyme to homogeneity. As the properties of the enzyme like overall charge, isoelectric pH, molecular size and shape differ with each lipase, the purification strategies also are different for different enzymes.

The most commonly reported chromatographic technique is ion exchange with the anion exchange resin Diethylaminoethyl (DEAE) cellulose or cation exchange resin Carboxymethyl (CM) cellulose. Gel filtration is the second most used chromatographic technique (Saxena, *et al.,* 2003b). Due to the surface hydrophobic patches on lipase structure hydrophobic interaction chromatography (HIC) can be considered effective for lipase purification (Dalal *et al.,* 2008; Chander and Shamsher, 2012; Trbojevic *et al.,* 2013). The commonly used hydrophobic adsorbents include butyl, octyl or phenyl

sepharose. A two step purification with ammonium sulfate precipitation and HIC was successfully conducted by Saxena *et al.,* (2003) and Salameh and Wiegel, (2007). Affinity and adsorption chromatography are used less frequently in the purification of lipases as they are comparatively expensive. Glycoprotein nature of lipases make it possible to use heparin and Concanavalin A (Con A) (Farooqui *et al.,* 1994) for the purification of lipases. Other reported strategies for lipase purification includes membrane processes (Sztajer and Bryjak 1989), liquid–liquid extraction of biomolecules employing reversed micelles (Leser *et al.,* 1993), immunopurification with monoclonal antibodies or affinity purified polyclonal antibodies (Bandmann *et al.,* 2000; Belguith *et al.,* 2013), aqueous two phase extraction (Gupta *et al.,* 1999), hydrophobic interaction chromatography employing epoxy-activated spacer arm as a ligand (Queiroz *et al.,* 1995) and polyethylene glycol–sepharose gel (Queiroz *et al.,* 1996) or poly(vinyl alcohol) (Battinelli *et al.,* 1996) polymers as column chromatography stationary phase. Conventional purification strategies are considered time consuming with lower yields and novel approaches based on purification in ionic liquids and purification based on lipase-lipase interaction are being considered recently (Nagarajan, 2012; Singh and Mukhopadhyay, 2012).

1.2.11. Magnetic nanoparticle immobilized lipase for biodiesel production

Immobilized enzymes are being used since 1916, when Nelson and Griffin (1916) discovered that invertase when absorbed to charcoal has the ability to hydrolyse the sucrose. Powel (1996) described enzyme immobilization as the physical confinement or localization of enzymes in a certain region of space with retention of their catalytic activity and which can be used repeatedly and continuously. Enhanced stability (Mateo *et al.,* 2007), repeated or continuous use and easy separation from the reaction mixture, improved enzyme activity are the major advantages of immobilized enzymes (Reetz, 1997; Huang *et al.,* 2003; Uwe, 2003; Elif and Mehmet, 2012). Different methods have been used for immobilization which can be broadly categorized as physical where weak interactions like hydrogen bonds, salt bridges and van der Waals forces exist between the support matrix and the enzyme and chemical where a covalent bonding is involved. The most frequently used immobilization methods include adsorption or deposition, entrapment or encapsulation in a polymeric gel, cross linking or covalent attachment of

the enzyme with the support material (Margarita *et al.,* 2012). No single method and support can be considered best for all enzymes. This is due to the versatility in enzyme nature and mode of action in various reaction conditions. It also depends upon the specific applications for which the immobilized enzymes are being used. The possibility of using magnetic fine particles for the binding of proteins was first reported in late 80's (Tamaura *et al.,* 1986; Mihama *et al.,* 1988; Koneracka *et al.,* 1999; Koneracka *et al.,* 2002). Magnetic nano particles activated with 3-amino propyl triethoxy silane and coupled with glutaraldehyde or silica particle was used for immobilizing enzymes, antibodies (Shinkai *et al.,* 1991; Roque *et al.,* 2009). Here covalent bond is formed between functional groups present on the surface of the magnetite particles and functional groups belonging to amino acid residues on the surface of the enzyme. Leakage of enzyme from the matrix used is often minimized with covalently bound immobilized enzymes (Cao, 2005). Localization of enzyme to the support material can enhance the substrate access and hence enzyme activity. But there are concerns in enzyme activity loss due to change in configuration because of the involvement of active site of the enzyme in bond formation (Elnashar, 2011; Yi-Yu *et al.,* 2013). Easy and fast separation of enzymes from the reaction mixture using an external magnetic field makes this an attractive method for immobilization compared to other methods. Primary reports on magnetic nano particle bound lipase with enhanced activity and stability includes Huang *et al.,* 2003. A reactive extraction process for biodiesel production using *Candida rugosa* lipase immobilized on magnetic nanostructures was described earlier by Dussan *et al.* (2007). Similar reports of successful transesterification reactions using used cooking oils and soybean oil with high binding efficiency and conversion rate are also there (Xie and Ma 2010; Kanimozhi and Perinbam, 2013)

1.2.12. Cloning of lipases from yeasts

It is reported that more than 50% of yeasts produce lipase isozymes encoded by different genes (Vakhlu and Kour 2006). More than one lipase encoding genes in a single organism perhaps have originated from gene duplication. A number of yeast lipases and their isoforms have been cloned and expressed (Vakhlu and Kour 2006). PCR methodologies based on specific (Xu *et al.,* 2010) as well as degenerate primers (Neugnot *et al.,* 2002; Bigey *et al.,* 2003; Thongekkaew and Boonchird 2007; Zhang *et*

al., 2007; Wang *et al.,* 2008) were used in majority of the successful cloning strategies. Degenerate primers were designed based on the consensus motiff (G-X-S-X-G) present in the active site which is conserved in most lipolytic enzymes. These sequences may have slight variations (Neugnot *et al.,* 2002; Bigey *et al.,* 2003) within each species and gene families. PCR methodology had also been used to prospect for novel lipase genes directly from environment DNA despite the low homology observed between lipases (Bell *et al.,* 2002) This was made possible by extensive analysis of conserved regions and careful primer designing based on oxy anion hole and active site of lipases. Amplification of lipase gene using primers designed corresponding to the peptide sequence after N terminal sequencing is also popular in the last decade (DeAngelis *et al.,* 2007). cDNA library construction followed by identification of lipase gene through colony hybridization or PCR using oligonucleotides designed based on the consensus sequence of the enzyme is yet another strategy used for lipase gene cloning in yeasts (Shimada *et al.,* 1989; Tsuyoshi *et al.,* 1997). Successful protocols were reported earlier to clone full length gene of enzymes (Morris *et al.,* 1998) and an improved genome walking technique based on cassette ligation was used to obtain complete gene sequence of lipase from Antartican marine bacteria (Acevedo *et al.,* 2008). Lipase gene cloning by short gun approach is also common (Ashton, 2013).

1.2.13. Metagenomic approach for novel biocatalysts

The term "metagenomics" was first used by Handelsman *et al.* (1998) and first appeared in publication in 1998. Metagenomics is the study of metagenomes, genetic material recovered directly from environmental samples. Early metagenomic studies conducted by Handelsman revealed that there are probably large groups of microorganisms in many environments that cannot be cultured and thus cannot be sequenced. His work revealed the concept of cloning the metagenome to access the collective genomes and the various biosynthetic machinery of soil microflora had been explored. Because only a tiny fraction of microbes from soil are readily cultured, soil might be the greatest untapped resource for novel chemistry. The extensive work on metagenomics through shot gun sequencing done by Venter *et al*. (2004) from the marine samples of Sargasso sea explored millions of unknown genes and around 150 novel bacterial phylotypes.

Widespread studies have been made from late 90s to investigate the "metagenome" which represents the genomes of uncultured microorganisms as a rich source for isolation of novel genes (Rondon *et al.,* 2000). Apart from prospecting for the desired catalytic activities, the advantage of this approach is that the already prepared environmental libraries can be employed for the screening of various other targets. The metagenomic approach thus provides an alternative way to access and exploit the immense pool of genes from microorganisms that have not been cultivated so far (Henne *et al.,* 2000)

In an effort to isolate novel genes from enormous and largely unexploited gene pools in uncultured microorganisms and/or those that are difficult to culture, the metagenomic library approach has been used successfully (Rhee *et al.,* 2005). Several different laboratories have successfully isolated novel genes encoding different enzymes and secondary metabolites from microbial communities and their metagenomes without cultivation of the microbes (Cottrell *et al.,* 1999; Henne *et al.,* 1999; Beja *et al.,* 2000; Henne *et al.,* 2000; Rondon *et al.,* 2000; MacNeil *et al.,* 2001; Uchiyama and Miyazaki 2009; Rabausch *et al.,* 2013; Wang *et al.,* 2014) The microbial niches studied were highly diverse and ranged from moderate environments, such as river soil (Henne *et al.,* 1999), to rather extreme environments, like the deep sea (Beja *et al.,* 2000). Among the novel genes isolated through metagenomic approach, there are also novel esterases and lipases (Henne *et al.,* 2000; Bell *et al.,* 2002; Voget *et al.,* 2003; Rhee *et al.,* 2005; Wang *et al.,* 2013; Peng *et al.,* 2014).

The studies done on metagenomics can be divided into three categories.

a. Studies employing function based screening of fosmid, cosmid or BAC (Bacterial Artificial Chromosome) derived metagenomic libraries (Neufeld *et al.,* 2008; Brazelton and Baross 2009; Martinez *et al.,* 2010).

b. Sanger sequencing derived shot gun metagenomic studies (Gianoulis *et al.,* 2009; Sebastian and Ammerman, 2009).

c. Next-generation sequencing derived shot gun metagenomic studies (Willner *et al.,* 2009; Tripp *et al.,* 2010)

The above 3 approaches were used in recent studies to explore the incredible biodiversity as well as novel unknown genes from different exotic locales far and wide (Thurber *et al.,* 2009; Wooley *et al.,* 2010; Gilbert, 2010). Metagenomic studies on eukaryotic haptophytes and picoplanktons were also reported (Liu *et al.,* 2009; Cuvelier *et al.,* 2010)

One of the biggest challenges in metagenomics lie in analysis of the obtained sequence data (Chen and Pachter, 2005). Development of new computation methods based on databases to analyze the shot gun sequencing data has been advancing in recent years (Meyer *et al.,* 2008; Brady and Salzberg 2011; Sharpton *et al.,* 2011; Segata *et al.,* 2012; Matsen and Evans, 2013). A newly introduced database Phylosift helps in phylogenetic analysis of microbial community structure directly from metagenomic sequence data (Darling *et al.,* 2014)

1.3. Objectives of the work

Lipases are hydrolases acting on acylglycerides and they hold tremendous potential for industrial exploitation. They are among the most used enzymes in industry due to their stability in organic solvents and their ability to catalyze a wide range of reactions with a high regio and stereo specificity combined with broad substrate specificity. Despite the commercialization of a large number of lipases from varying sources, there still exists a demand for novel lipases due to the tremendous amount of unique bio-catalytic possibilities where these enzymes can be employed. Considering the ever increasing demand on novel lipase activities especially for applications which do not have an enzymatic route, it becomes evident that any quest for novel lipases is largely welcome. The major objective of the work was to explore the cultivable and uncultivable biodiversity for novel esterases/lipases through classical and metagenomic approach. The objectives of the present study included

1. Screening of microbes through conventional methods as source for novel esterases/lipases and isolation of potent strain(s) capable of producing solvent, temperature and halo tolarent lipase/esterase
2. Optimization of the fermentation production of esterase/lipase under submerged fermentation employing using statistical experiment design.
3. Purification and characterization of esterase(s) obtained through conventional screening.
4. Collection of environmental soil samples from exotic locales and isolation and purification of environmental DNA.
5. Construction of large insert metagenomic libraries in Fosmid and BAC.
6. Function based screening for esterase(s)/lipase(s) by assaying lipase activities.
7. Isolation and purification of novel esteraces(s)/lipase(s) obtained through metagenomic approach.
8. Characterization of novel lipases for position specificity, fatty acid chain length, tolerance for temperature, pH and salinity.
9. Possible applications of novel lipases.

Chapter 2
Materials and Methods

2.1. Microorganisms and culture conditions

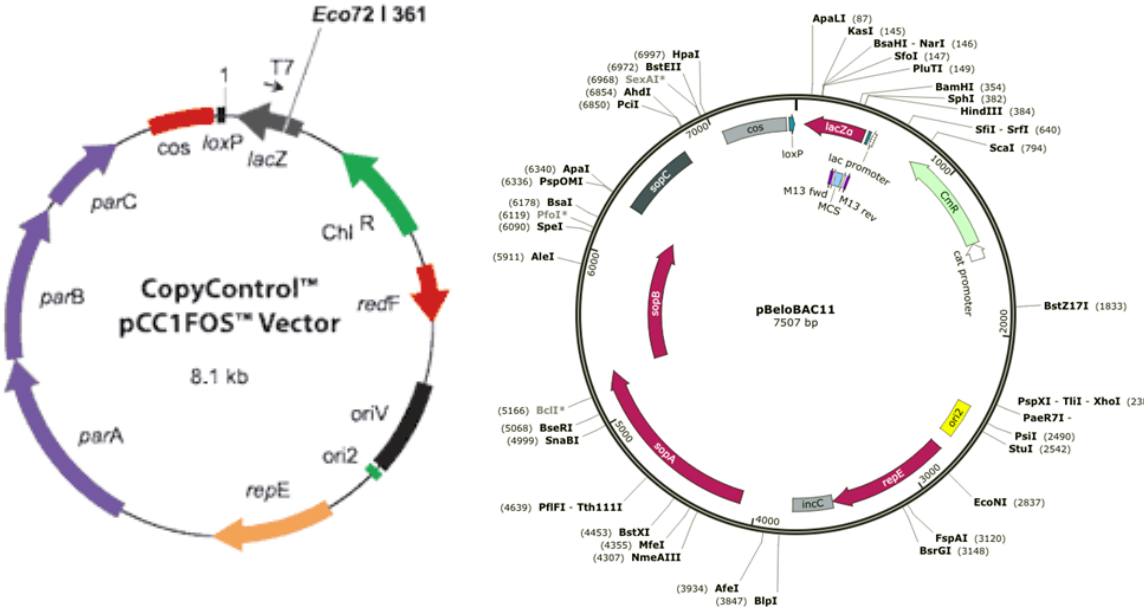
The esterase producing strain used for the current study was isolated through an aerial sampling at the CSIR- National Institute for Interdisciplinary Science and Technology and was identified by as *Pseudozyma* sp. The culture showed brilliant fluorescence on the Rhodamine-Tributyrin Agar plate and on initial trials showed good esterase activity. The culture was deposited in NII culture collection with accession no. NII08165. It was maintained on potato-dextrose agar slants and was sub cultured fortnightly.

Escherichia coli strains DH10B™ and EPI300 used for metagenomic library preparation were grown on Luria Bertani Agar at 37 °C and were stored at 4 °C for short term preservation. The cultures were preserved as 50% glycerol stocks at -80 °C for long term storage. The bacterial strains and vectors used in the present study are listed in Table 2.1

Table 2.1. List of Bacterial strains and vectors used in the present study

Bacterial strains (*E.coli)*	Characteristics	Source or Reference
DH10B™	*F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139Δ(ara, leu)7697 galU galK λ- rpsL nupG*	Life Technologies
EPI300™	*F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL (Str^R) nupG trfA dhfr*	Epicentre
Vectors		
pCC1FOS™	*Chloramphenicol resistance marker, E coli F factor based single copy origin of replication, oriV high copy origin of replication, Phage λ cos site, Phase PI loxP site, T7 promoter, lacZα, 8139bp*	Epicentre (Fig 2.1A)
pBeloBAC™	*T7 promoter, SP6spromoter, lacZα,cat, repE, Ori2, sopA, sopB, sopC, cos site, lox P site*	New England Biolabs (Fig 2.1B)

Figure 2.1. Vector maps of pCC1FOS™ (Epicentre) and pBeloBAC™ (NEB)



2.2. Medium for Enzyme production

Growth and enzyme production was carried out in a basal mineral salts medium with the following composition (Table 2.2)

Table 2.2. Composition of Basal Mineral Salts Medium (BSM)

Components	g/l
Urea	1.3
$(NH_4)_2SO_4$	2.0
KH_2PO_4	5.0
$MgSO_4.7H_2O$	2.0
Peptone	1.0
Glucose	10.0
Olive Oil	Variable up to 1.0%

The pH of the medium was adjusted with 1N HCl or 1N NaOH wherever required

2.3. Preparation of inoculum

For preparation of inoculum, a loop full of culture was inoculated into 3ml of the sterile growth medium which was BSM in the case of *Pseudozyma* sp. and LB broth in the case of recombinant *E. coli*. Incubation was carried out at either 30 ^{o}C (*Pseudozyma*), or 37

°C (*E.coli*) for 12h. One milliliter of this culture was transferred to 100 ml of the same medium but supplemented with appropriate amount of olive oil or tributyrin in 500ml Erlenmeyer flasks. The seed culture was grown with 200 rpm agitation for up to 72h at the respective incubation temperature. Growth was monitored as absorbance of the culture medium at 600nm and a 1.00 OD culture was used for inoculation at desired level.

2.4. Production of Esterase

Submerged Fermentation (SmF) for enzyme production was carried out in 500 ml Erlenmeyer flasks having 100 ml of BSM (*Pseudozyma sp*) or LB broth (*E. coli*) with appropriate amount of olive oil. The contents of the flask were mixed thoroughly and the flasks were sterilized by autoclaving for 15 minutes at 121 $^{\circ}$C and 15 lbs pressure. After cooling, the flasks were inoculated with 1 ml of a 1.0 OD inoculum prepared as under section 2.3 (unless otherwise specified). The flasks were incubated at 30 ± 2 °C (*Pseudozyma* sp) or 37 ± 1 °C (*E coli*) and 200 rpm agitation for the specified time interval. The culture broth after fermentation was centrifuged at 10000 rpm for 15 min at 4 ^{o}C and the supernatant was used as the crude enzyme preparation.

2.5. Analytical methods

2.5.1. Enzyme assay

Esterase activity was measured as the ability to hydrolyze para nitrophenyl palmitate (pNPP) by a modified method of Gupta et al (2006). Appropriate volume of enzyme was incubated with 1mM solution of pNPP in buffer A (50mM Tris, pH 8, containing 50mM NaCl, 0.4% Triton X-100 and 0.1 % Gum Arabic). The reaction mixture was incubated at 50 °C for 30 min and absorbance readings were taken at 405nm in a UV-Visible spectrophotometer. Standards were run with appropriate concentrations of para nitro phenol (pNP). One unit of esterase activity was defined as the amount of enzyme liberating 1uM of pNP per milliliter per minute.

2.5.2. Protein assay

Protein assay was done using the Bradford's reagent according to the method of Bradford (1976) and was expressed as mg/ml.

2.6. Electrophoresis and Zymogram analyses

2.6.1. Poly Acrylamide Gel Electrophoresis (PAGE) and Zymogram analyses

The molecular weight as well as the homogeneity of the protein was determined by Sodium Dedecyl PAGE (SDS PAGE) as well as Native PAGE using the method of Laemmli (1970). Gels with 12% strength of acrylamide were used for the electrophoresis. Protein in the samples was estimated by Bradford's method and samples were normalized for protein content before loading. Gels were run on a Biorad Protean Xi® vertical electrophoresis unit with a constant voltage of 80V. After completion of the electrophoresis, the gels were washed once in distilled water and were incubated with 0.1 M Methylumbelliferyl Butyrate (MUB) solution in Tris buffer (0.1 M, pH 8.0) for 10 min at room temperature (30 ± 2 °C). Esterase activity was visualized as blue–green fluorescence under long wavelength UV trans-illumination. Gels were photographed using an imaging system (Syngene-GBox, UK), to avoid differences in lighting and exposure. After this the same gels were washed thrice in distilled water and were used for silver staining according to the protocol of Blum et al (1987). In brief, the gels were fixed with 50 % methanol/10% acetic acid for 30 min followed by 50% methanol for 15 min and was washed with deionized water. The gels were sensitized by incubating in 0.02% sodium thiosulfate for 1-2 minutes and were rinsed again. The treated gel was then incubated with 0.02 M silver nitrate solution for 20 min followed by rinsing in distilled water. The gels were developed in a solution of 0.04% formalin in 3% sodium carbonate, and the reaction was terminated with Na_2-EDTA (14g/L). Gels were photographed using an imaging system (Syngene-GBox, UK) for documentation.

2.6.2. Agarose Gel Electrophoresis

Agarose Gel electrophoresis of DNA was conducted in a Biorad Horizontal Gel apparatus. 1.0 % Agarose gel was used for PCR amplicons and 0.8% was used for Genomic DNA unless otherwise specified. Ethidium bromide was included in the gel for fluorescent visualization of DNA fragments under UV light. On every gel 0.5µg of 1kb DNA ladder (Fermentas, USA) or another appropriate ladder was run as a molecular weight marker for determination of the approximate size of DNA fragments. Nucleic acid bands were visualized under long wavelength UV (302nm) and were photographed using an imaging system (Syngene-GBox, UK) for documentation.

2.7. Yeast chromosomal DNA isolation

Chromosomal DNA was isolated from yeast using a modified protocol of Hoffman (Hoffman and Winston, 1987). Aliquots of 1.5 ml of the overnight grown cultures were centrifuged at 10,000 rpm for 5.0 min. Cell pellets were then resuspended in 200 µl of lysis buffer [2% Triton X-100, 1% SDS, 100 mM NaCl, 1mM EDTA, 10 mM Tris-HCl (pH 8.0)]. The tubes were placed in a -80 °C freezer for 2.0 min and were then immersed in a 95 °C water bath for 1.0 min to thaw quickly. The process was repeated 2-3 times and the suspension was vortexed vigorously for 30 seconds. Phenol: Chloroform mixture (200µl) was added to the tubes, vortexed and was then centrifuged room temperature at 20000g for 3.0 min. The aqueous layer was transferred to a tube containing 400 µl of ice-cold 100% ethanol. The samples were allowed to precipitate for 5.0 min at room temperature and then centrifuged for 10 min at 16,000 rpm. DNA pellets were washed with 0.5 ml of 70% ethanol followed by air drying. DNA was resuspended in 20 µl TE buffer (10 mM Tris, 1 mM EDTA (pH 8.0)).

2.8. Plasmid isolation from metagenomic clones

Plasmids from BAC as well as fosmid libraries were isolated according to the alkaline-lysis method proposed by Birnboim and Doly (1979). Briefly, aliquot of 1.5 ml overnight culture in micro centrifuge tube was pelleted and 100µl of Solution 1 (50mM glucose, 5mM Tris Cl (pH 8), 10mM EDTA (pH 8)) was added. The mixture was vortexed well and 200 µl of Solution 2 (0.2 N NaOH, 1% w/v SDS) was added. Tubes were gently mixed by inverting and were kept for 15 min at room temperature. Hundred and fifty micro liters of Solution 3 (5M Potassium acetate, glacial acetic acid) were added to the tube and were kept in ice for 15-20 min. The tubes were centrifuged at 13000 rpm for 15 min and the supernatant was gently transferred to a new tube. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed well by gentle inversions and centrifuged at 13000 rpm for 15 min to recover the aqueous phase. The aqueous phase was transferred carefully to a fresh tube and 0.6 V of iso-propanol was added. The tube was kept in ice for 20 min to precipitate the DNA. DNA was recovered by centrifugation at 13000rpm for 10 min. The cell pellet was washed with 70% ethanol and after air drying was dissolved in TE buffer (10 mM Tris, 1 mM EDTA (pH 8.0) or distilled water.

2.9. Production of electrocompetant cells

Electro competent DH10B *E. coli* cells were produced by repeated washing with 10% glycerol at 4°C based on a modified protocol of Sambrook and Russel (2001). In brief, 100 ml of a 2.0h old culture (0.7 OD) was centrifuged at 3000 rpm for 7.0 min at 4 °C. The pellet was kept on ice for 5.0 min and was resuspended in 30 ml of 10 % ice cold glycerol. The suspension centrifuged as above and was resuspended in 15ml of 10% ice cold glycerol. The process was repeated again by suspending the pellets in 5 ml and finally in 1ml of ice cold glycerol (10%). The suspension was aliquoted into pre-chilled Eppendorf® tubes and was stored at -80 °C until used.

2.10. General Molecular Biology Techniques and Software

All DNA manipulations, buffer preparations when required and general molecular biology operations were performed following the standard methods as specified in Sambrook and Russel (2001). Primer designs were performed using FAST PCR (Kalender et al, 2014) and degenerate primers were designed using the CODEHOP web server (Rose et al, 2003). Sequence alignment and database search was performed using NCBI BLAST (Altschul et al, 1990). Nucleic translations were performed using the Expasy web server (Gasteiger et al, 2003). Protein Family and domain searches were performed using the Interpro web server (Apweiller et al, 2001)

2.11. Experimental Data Analyses

All experiments were performed in triplicates unless otherwise indicated/implied (eg Molecular Biology Experiments, Electrophoresis, Chromatography etc) and numerical data were statistically analyzed using Microsoft Excel®. DOE data were analyzed using Design expert (Stat Ease Corp, USA). Graphical representation of data was generated using either SigmaPlot (Systat Software, USA) or Microsoft Excel ®.

Chapter 3

Screening and selection of a novel microbe for esterase production

3.1. Introduction

Microorganisms can be considered as a treasure trove of useful enzymes. Majority of biologically relevant processes for making chemicals, drugs and pharmaceutical molecules, biofuels and many other products make use of microbial enzymes. The potential value of an enzyme cannot be determined easily and isolation of a novel potent enzyme always requires tedious and stringent screening strategies that may meet our enzyme requirements. Function based screenings as well as sequence homology based screening methods are the two different approaches conventionally used in screening of lipases. Generally, function based screening is preferred over sequence based screening strategies since it enables isolation of an enzyme which is functionally active rather than just identifying a potential enzyme which may not be expressed or may be dysfunctional, though having sequence similarity to the target enzyme. This is especially true in the case of screening metagenomic libraries. Agar Plates containing tributyrin emulsified with gum arabic and egg yolk are conventionally used for esterase/lipase screening (Mourney and Kilbertus, 1976; Kok *et al.*, 1993). It is known that esterases/lipases are hydrolases that cleave ester bonds. When carboxyl esterases preferentially hydrolyze water soluble esters and triacylglycerols containing short fatty acids, lipases cleave triacylglycerols containing medium as well as longer chain fatty acids (Pandey *et al.*, 1999; Jaeger *et al.*, 1999; Jaeger and Eggert, 2002; Fredrik & Sara, 2007). Lipases/esterases are highly unique in its biocatalytic reactions and can be considered as potent candidates in industrial applications (Hasan *et al.*, 2006). These enzymes exhibit broad substrate specificity along with its high stereo and regio specific characteristics (Bornscheuer and Kazlauskas, 1999; Liese et al, 2006). Therefore, despite a large number of lipases/esterases being commercialized, there still exist demands for novel lipases/esterases due to the tremendous amount of biocatalytic possibilities where this enzyme can be employed

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3.2. Materials and Methods

3.2.1. Sample collection and primary screening for Esterase producers.

Microbes were isolated from sludge samples collected near the effluent outlet of an oil mill. One gram of the sludge was suspended in 100 ml sterile distilled water and was then serially diluted in sterile saline. The diluted samples were then plated on nutrient agar plates and were incubated at 30 ± 2 °C for 24-48h. The colonies obtained were purified by streak plating on nutrient agar plates. Colonies obtained through aerial sampling from the lab were also included in the screening. Isolated pure colonies were spotted on tributyrin-rhodamine (TBR) agar plates and incubated at 30 °C for 4 days to test for esterase/lipase production. Lipase producing organisms display an orange fluorescent halo around their colonies under UV light.

3.2.2. Esterase production

Lipase positive culture that showed maximum fluorescence in TBR agar plates were selected for further studies. The culture was identified as a species of *Pseudozyma* by molecular identification and was deposited in the NII culture collection (CSIR-NIIST, Thiruvananthapuram, India) with the accession number NII 08165. The culture was used for enzyme production by submerged fermentation (SmF) following the protocol described under section 2.4. Culture supernatants obtained after centrifugation at 10,000 rpm for 15min at 4 °C were used as crude enzyme preparation. Esterase activity in the enzyme preparations were assayed as described in section 2.5.1.

3.2.3. Native PAGE and Zymogram analysis

Enzyme samples were assayed for protein content by Bradford's method (Bradford, 1976). The crude enzyme preparations were concentrated by vacuum concentration using a centrifugal vacuum concentrator (Eppendorf, Germany), normalized for protein content and was subjected to Native Polyacrylamide Electrophoresis (Native PAGE) as outlined under section 2.6. Esterase activity of the electrophoretically separated protein bands were monitored by Zymogram analysis using Methylumbelliferyl Butyrate (MUB) as substrate (section 2.6.1).

3.2.4. Identification of microbial strains.

Morphological features were studied as well as rRNA sequence analyzes were performed for identification of the selected microbial culture- isolate NII 08165.

3.2.4.1. Morphological identification of NII08165

For morphological examinations, actively growing yeast cells were observed under phase contrast microscope (Leica DM 2000, Germany). Images were captured in an attached digital camera (Leica DFC 295) for documentation. Morphological features were compared against the standard features used for differentiating yeasts.

3.2.4.2. Molecular identification of NII08165

Identity of the yeast was also confirmed by amplification of rRNA Internal Transcribed Spacer (ITS), which is widely used in taxonomy and molecular phylogeny as it has a high degree of variation even between closely related species. Yeast DNA was isolated using the modified protocol Hoffman and Winston (1987) as described under section 2.4. The ITS region was amplified using primers ITS1 (5' -TCCGTAGGTGAACCTGCG G-3') and ITS4 (5' -TCCTCCGCTTATTGATATGC- 3') (White *et al.*, 1990). PCR reactions contained 0.5 units of *Taq* DNA polymerase, 1x *Taq* buffer, 200 μM of each dNTP, 2.0 μM $MgSO_4$, 0.2 μg genomic DNA, and 0.5 μM forward and reverse primers. Reaction conditions for PCR were an initial 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 1 min; and a final extension step at 72 °C for 10 min. An Eppendorf ® gradient PCR system was used for the amplification. The PCR amplicon was sequenced and BLAST analysis was performed (Atschul et al, 1990). The sequences were aligned using Clustal W software (Thompson *et al.*, 1994). The phylogenetic analysis was performed with MEGA4 software (Tamura *et al.*, 2007).

3.2.5. Enzyme Profile

The isolate NII-08165 was checked for the production of other enzymes like amylase, xylanase, cellulase, pectinase and protease to obtain a general enzyme profile of the organism. In brief, the screening was done by conducting agar plate assay in standard media containing respective substrates and checking for the zone of clearance around the colonies due to utilization of the particular substrate.

3.2.5.1. Amylase

Screening for amylase production was carried out by starch agar plate assay (Akpan *et al.*, 1999). Culture was grown plates with basal mineral salts medium (section 2.2) containing 1% soluble starch, incubated at 30°C for 72h. The plates were stained with grams iodine solution for 15 min, and washed with water to remove excess color.

3.2.5.2. Xylanase

Xylanase production by the yeast was tested by the congo red test (Teater and Wood, 1982). Microbial culture was allowed to grow on the basal mineral salts medium containing 0.25 % xylan for 16-24 h at 30 °C. Ten milliliters of a 1mg/ml solution of congo red was added and the plates were incubated for 15 min to stain xylan. Plates were destained using 1.0 M NaCl.

3.2.5.3. Cellulase

The protocol used for testing cellulase production was similar to that for xylanase (Teater and Wood, 1982). Culture was grown in basal mineral salts medium containing 1% carboxy methyl cellulose, and cellulase producers were screened using congo red staining to detect clearance zones as outlined above (section 3.2.5.2)

3.2.5.4. Pectinase

Pectinase production of the culture was analyzed using a modified protocol Cappuccino and Sherman (2007). Agar plates with the basal mineral salts medium containing 0.5 % pectin were used for screening. After incubating the culture for 24 hours, plates were tested for pectinase activity by flooding it with iodine solution and observing the clearance zone around the colonies.

3.2.5.5. Protease

Protease production of NII08165 was tested in 1% casein agar plate (Sharmin *et al.*, 2005). Culture was incubated for 24h at 30 °C and was observed for clearance zone around colonies.

3.3. Results and Discussion

3.3.1. Screening and isolation of lipase producing cultures.

A total of about 76 pure cultures were isolated from the effluent sludge and through aerial sampling. The isolates included mostly bacteria but also contained fungi. Aerial sampling provided few yeast like isolates in addition to bacteria and fungi. While the fluorescence reaction worked fine it was noted that the TBR agar assay resulted in a spreading of cultures in most of the isolates due to the spreading of Tributyrin over the agar surface. The isolates were designated as BTLn where "n" is incrementing from 1 to 76. Out of the 72 pure culture isolates tested for esterase activity, 13 were positive. This included bacteria (BTL 5a, 5a (1), 9, 11, 12, 15a, 15f, 28, 36), fungi (BTL 6, 14), and yeasts (BTL1, 29) (Fig 3.1). The yeast like culture BTL1 that showed the highest fluorescence in tributyrin-rhodamine (TBR) agar plates was selected for further studies. Lipase production by the culture was tested by performing the esterase assay and zymogram analyses.

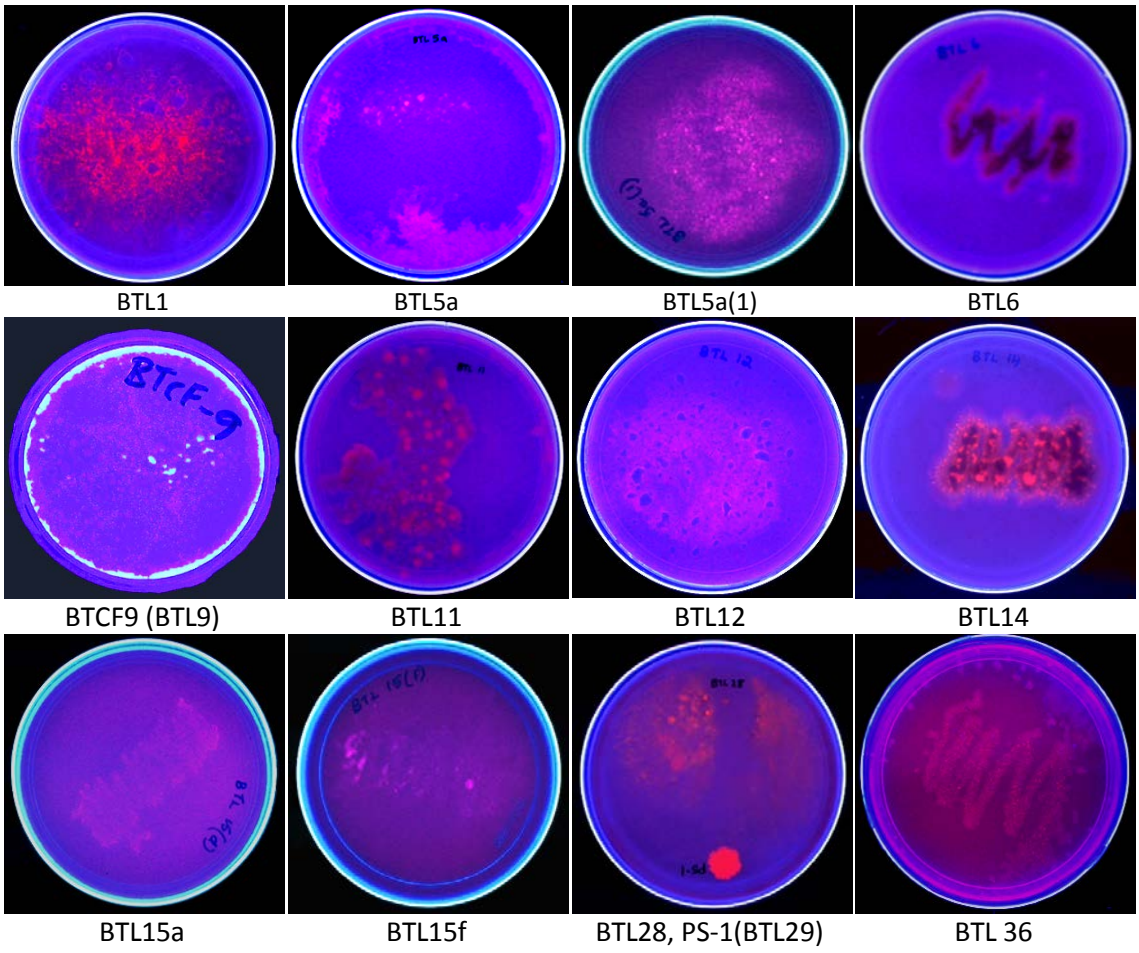
3.3.2. Lipase production by BTL1

Lipase production by the isolate BTL1 was tested under submerged fermentation (SmF). The culture was grown in the basal mineral salt medium supplemented with olive oil as described under section 2.4. The culture supernatants were collected from 24h onwards till 96h and were assayed for esterase activity using the pNPP assay. The results presented in Table 3.1 indicated that the culture produced esterase starting from 48h and the yield of esterase increased with the duration of incubation.

Table 3.1. Evaluation of Esterase production by BTL1

Duration of Incubation (h)	Esterase Activity (U/ml)
24	75.36
48	171.48
72	187.51
96	300.00

Fig. 3.1. Screening of pure culture isolates for esterase activity by Tributyrin-Rhodamine agar method



3.3.3. Zymogram analysis

Zymogram analysis was performed for the concentrated culture supernatant of BTL1 for detecting the esterase protein. Activity staining of the Native PAGE gel with methyl umbelliferyl butyrate showed more than one fluorescent bands indicating the possible presence of more than one lipase/esterases. More than one lipase is reported in many yeasts (Kirk & Christensen, 2002) and it is also reported that about 50 % of the reported lipase producing yeasts produce it in the form of various isoforms (Vakhlu & Kour, 2006). At least 3 different bands of activity as detectable in the Zymogram analysis of BTL1 culture supernatants indicating that the isolate produced a minimum of 3 lipases/esterases or 3 different isoforms of the enzyme which were secreted (Fig 3.2)

Fig.3.2. Zymogram analysis of the culture supernatant of BTL1 showing lipase activity

Lane Information

1 1µg total protein

2 5µg total protein

3 15µg total protein

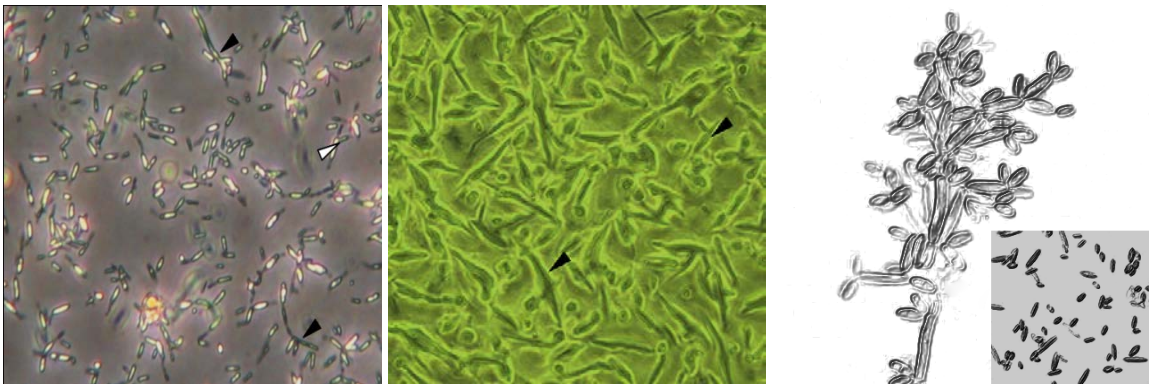
20ul each of concentrated culture supernatants with protein concentrations adjusted as required were loaded on to each well. Markers not included as this is a Zymogram. Esterase activity bands were detected as fluorescence on release of methylumbelliferone from MUB by cleavage of it due to esterase activity.

3.3.4. Identification of the isolate BTL 1

3.3.4.1. Morphological identification

The colonies of the isolate BTL1 had typical features of yeast when cultured on agar plates. The culture was cultivated in Yeast Extract Peptone Dextrose /YPD medium (HiMedia®, India) and the log phase cultures were observed under a phase contrast microscope to study the morphological features. The isolate displayed characteristic morphological features of the basidiomycetous yeast *Pseudozyma*. The colonies were pale white to cream in color on YPD agar plates, with the cells showing a cylindrical to fusiform shape. Conidiogenesis was on short polar denticles and the blastoconidia were fusiform. Proliferation was sympodial (Fig 3.3). Based on these features it was speculated that the isolate could be a *Pseudozyma* strain and the isolate was deposited in the NII culture collection at CSIR-NIIST with an accession number of NII 08165. Further identification of the culture was performed by rDNA sequence analyses.

Fig.3.3. Morphological features of isolate BTL1 under microscope



A: Phase contrast image of BTLI cells showing elongated cylindrical and fusiform cells. Hyphae with sympodial proliferation is shown by black arrowheads and polar conidiogenesis on short denticles is indicated by white arrow head

B: Phase contrast image showing fusiform blastoconidia (Arrow heads)

C: Representative image of *Pseudozyma antartica* showing hyphae and chains of blastoconidia. Inset – fusiform blastoconidia. Image adapted from Wei et al, 2005 (For comparative purposes only)

3.3.4.2. Molecular identification of isolate NII 08165 (BTL1)

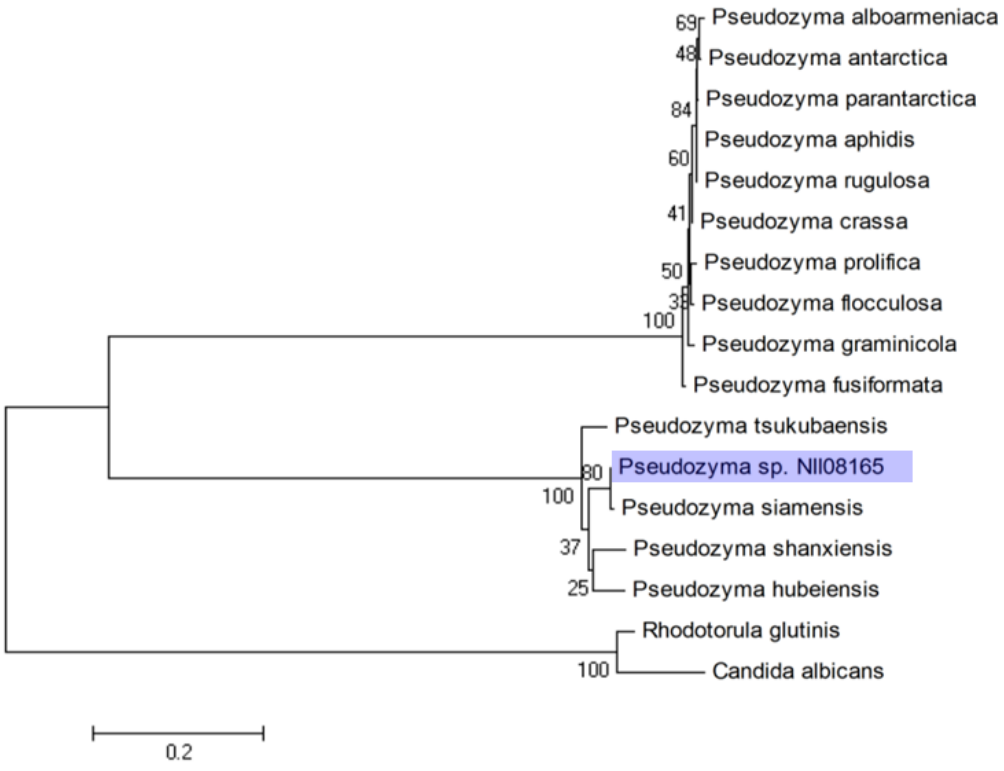
The yeast isolate NII 08165 speculated as a *Pseudozyma* was sent for molecular identification using rRNA sequence analyses to Agharkar Research Institute Pune and the ITS sequence amplification, sequencing and analysis was done at CSIR-NIIST. The analyses based on SSU and LSU rRNA done by Agarkhar institute confirmed that the isolate is indeed a strain of Pseudozyma with 97 % similarity with *Pseudozyma bandoni*. *Pseudozyma* anamorphic *Ustilaginaceous* yeast has about 9 species as mentioned in the *Dictionary of Fungi* (Kirk et al, 2008). A similar result was observed with ITS sequence analysis. The nucleotide sequence of ITS1-5.8s-ITS4 amplified from *Pseudozyma* sp NII 09165 was 614bp long (Fig 3.4). The sequence was deposited in Genbank with an accession number JN969989 (Sukumaran et al, 2011)

Fig. 3.4. Partial sequence of the ITS region of isolate *Pseudozyma* sp. NII 08165

>gi|377550372|gb|JN969989.1|
AACTTGAGCTACCTTTTTTAACACGGTTGCATCGGTTGGGCCTGTCAAATAG
CGCGGACGCGTTCCAAGCTACGAGACGGGTTCAACACTTTTACCAAAACACT
TTTGATGACTTAGGATTTGAATGATAAAAGTTCATTTTTAATAATGAAACCG
ACTGGTAATGCGGTCGTCTAATTTTAAAAACAACTTTTGGCAACGGATCTCT
TGGTTCTCCCATCGATGAAGAACGCAGCGAATTGCGATAAGTAATGTGAATT
GCAGAAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCCGGCAGATCT
AATCTGGGGAGCATGCCTGTTTGAGGGCCGCGAATTGTTTCGAACAACTGCT
TTTTTTAATTAAGAAGAAGTTGGATCGGTATTGAGGGTTTTGCCATTCACCG
TGGCTCCCTCGAAATGCATTAGCGCATCCATTTGATAGGCAAGACGGACGAA
AGCTCACTTTCGCTCTCTCTTCCCTGCCGGGTTTTGATAATATCAGGACTTC
GGAGAGGTTGAGTTGGGTACGAGCTGGAGCAACGGCTTGCTGTTTGGAGTGC
TTCTGAAACCCGCCCATGTCGAGCTTTATTGCTCGAAAGGGA

The results of phylogenetic analysis performed with MEGA 4 software is shown below (Fig 3.5). Results confirmed the strain as *Pseudozyma sp*.

Fig. 3.5. Phylogenetic tree of *Pseudozyma* sp NII 08165



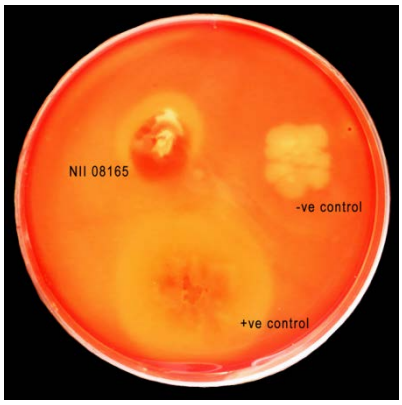
3.3.5. Enzyme profile

Enzyme profile of *Pseudozyma* sp NII 08165 was evaluated by plate assays to determine the ability of the yeast to utilize major carbohydrates and protein. Agar plate assays indicated that the culture was restricted in its carbohydrate utilization and was detected positive only for xylanase among the carbohydrate utilization enzymes tested (Table 3.2). The yeast was also positive for protease production detected by clearance zone in casein agar plates (Fig 3.6)

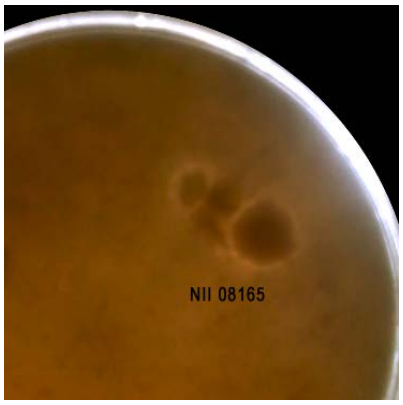
Table 3.2. Enzyme profile of *Pseudozyma* sp NII 08165

Enzymes	Plate assay
Amylase	_
Xylanase	+
Cellulase	_
Pectinase	_
Protease	+

Fig.3.6. Plate assays demonstrating the enzyme production profile of *Pseudozyma* sp NII 08165



A: Xylanase production indicted by clearance zone in Xylan supplemented medium



B: Protease production indicated by halos around colonies in casein agar

While the genus *Pseudozyma* is not well known for utilization of complex carbohydrates, certain strains like *P. antartica* are known to assimilate soluble starch (Goto et al, 1969, Kurtzman & Fell, 1998). Also while some strains do utilize glucosamine and xylose, utilization of chitin and xylan are not reported. The production of xylanase by this strain could be a unique feature which might provide advantage for this culture to thrive on plant material.

3.4. Conclusion

Screening for lipase/esterase producing cultures from oil factory effluent sludge and aerial samples resulted in the isolation of 72 isolates including bacteria, fungi and yeasts. The isolate BTL1 which showed highest production of esterase in plate assays were tested for esterase production by pNPP assay and was found to produce ~300U/ml of enzyme under SmF after 96h of incubation in olive oil containing medium. The culture was identified by morphological and molecular methods as a species of the basidiomycetous yeast genera *Pseudozyma* and was deposited in the NII culture collection with accession number NII 08165. The yeast produced at least 4 different esterases which were detected by a Zymogram analysis. The culture was also positive for xylanase and protease, the former a rather unusual feature for the genera *Pseudozyma*. The esterase titers were high indicating the potent use of this culture for production of the enzyme and hence the culture was selected for further studies.

Chapter 4

Fermentative production of esterase from *Pseudozyma* sp NII 08165

4.1. Introduction

The success of any fermentation process relies significantly on the characteristics of the production strain involved in the process. It is highly desirable to use a production strain which is high yielding and stable and capable of forming the product in a reasonable time frame. The enzyme productivity of natural microorganism can be increased to a certain extent by selecting an appropriate fermentation technique and optimization of the culture conditions. Submerged fermentation (SmF) has been a preferred method over the last two decades for the industrial production of enzymes in terms of space, labor and productivity (Ruuter *et al.*, 2002). SmF also provide the advantages of better sterility, heat and mass transfer, easiness of process monitoring and automation.

Process optimizations help to improve the productivity and also help to understand the effects of various media components and other physicochemical parameters on fermentation. Process optimizations which includes the media composition, culture conditions like temperature, pH, oxygen tension, aeration, agitation and type of cultivation etc plays a major role in enhancing enzyme yield. The need to achieve higher titers of enzymes, faster production rates and improved control over culture conditions among several other requirements for large scal3e production makes this process inevitable in industries (Ricardo *et al.*, 2003). In the conventional method for the optimization of enzyme production, the “one variable at a time” approach is used, which involves changing one parameter at a time while keeping the other entire parameters constant (Greasham and Inamine, 1986; Duan *et al.*, 1994). But this process is cost, labor and time intensive, and also does not consider the interaction between different variables. An alternative and more efficient approach is the use of statistical methods. Several statistical methods ranging from two factorial to multi factorial designs are available (Berenson *et al.*, 2011). Plackett & Burman design (Plackett and Burman, 1946) and Response Surface Methodologies (Myers *et al.*, 2009) are employed for identifying the important parameters in the fermentation process and optimizing these for obtaining maximum enzyme production.

When the parameters affecting the process in consideration are large as typical for fermentation processes, classical strategies to optimize the process include a two step method where the first method, typically a fractional factorial design like the Plackett & Burman (1944) method is used to screen the parameters for identifying the parameters. Once identified, these (significant) parameters are then optimized using response surface methodologies (Gangadharan *et al.,* 2008, Job *et al.*, 2010).

A basidiomycetous yeast culture capable of producing high titers of esterase was isolated at the Centre for Biofuels, Biotechnology division, CSIR-NIIST and was identified as a strain of *Pseudozyma* sp. (NII 08165). The objective of current study was to determine the effect of various media components and culture parameters on esterase production by this yeast using a fractional factorial Plackett and Burman design (Plackett and Burman 1944) and to optimize the levels of most significant parameters using a Response Surface – Box Behnken method (Box Behnken, 1962).

4.2. Materials and Methods

4.2.1. Strain and initial cultivation conditions.

Pseudozyma sp NII 08165 which was selected based on the TBR agar screening and esterase production testing by pNPP assay was used for production of the enzyme. The culture was maintained on potato-dextrose agar slants and was sub cultured every two weeks. The basal medium used for inoculum development and enzyme production contained in g/L $(NH_4)_2SO_4$-2, KH_2PO_4 -5, $MgSO_4.7H_2O$ -2, Peptone -1 and D-Glucose-10. Medium pH was adjusted to 6.00. The seed culture was grown at 30 ^oC with 200 rpm agitation. A 1.00 OD culture was used for inoculation at desired level.

4.2.2 Enzyme Assay

Lipase activity was measured as the ability to hydrolyze para nitrophenyl palmitate (pNPP) by a modified method of Gupta et al (Gupta *et al.,* 2006) as described in section 2.5.1. One unit of lipase activity was defined as the amount of enzyme liberating 1µM of paranitrophenol (pNP) per milliliter per minute.

4.2.3. Optimization of parameters affecting esterase production

Optimization of parameters for esterase production was performed in two stages. Initially 7 variables were screened using a fractional factorial design to identify the parameters which significantly influenced enzyme production and in the second stage the levels of these parameters were optimized using a response surface design.

4.2.3.1. Screening of parameters affecting esterase production by fractional factorial design

The components of medium used for esterase production and the important physical parameters affecting it was screened by a Plackett & Burman experimental design (Plackett and Burman, 1946) with 7 variables at two levels in a total of 8 experimental runs. The parameters were evaluated at two levels: a higher level designated as +1 and a lower level designated as -1. The actual and coded values are given in Table 4.3. The design matrix for the experiment was generated with the DOE software - Design Expert® (Stat-Ease Corp, Minniapolis, USA) and is given in Table 4.1.

Table 4.1: Actual levels of variables tested with the factorial design and their effects on esterase production

Variable Code	Parameter name	Low level (-1)	High Level (+1)
X_1	Glucose (% w/v)	0.5	1.0
X_2	Peptone (% w/v)	0.6	1.2
X_3	Olive oil (% v/v)	0.1	0.3
X_4	Inoculum (% v/v)	1.0	2.0
X_5	pH	4.0	6.5
X_6	Time (h)	96	120
X_7	$(NH_4)_2SO_4$ (% wt/v))	0.4	1.0

The parameters tested were: Glucose concentration, Peptone concentration, Olive oil content, Inoculum level, pH of medium, Incubation time and Ammonium sulfate concentration. The variables were tested at two levels: a higher level designated as +1 and a lower level designated as -1. The actual design matrix for experimentation is given in Table 4.2.

Table 4.2. Coded Plackett & Burman design matrix for the screening of variables influencing esterase production from *Pseudozyma*

Std order	X_1	X_2	X_3	X_4	X_5	X_6	X_7
1	-1	-1	-1	1	1	1	-1
2	1	-1	-1	-1	-1	1	1
3	-1	1	-1	-1	1	-1	1
4	1	1	-1	1	-1	-1	-1
5	-1	-1	1	1	-1	-1	1
6	1	-1	1	-1	1	-1	-1
7	-1	1	1	-1	-1	1	-1
8	1	1	1	1	1	1	1

Please refer table 4.1 for actual values of the levels of each parameter under study

Experimental runs were performed according to the design and the response (Enzyme activity) was recorded. A factorial model was fitted for the main effects using Design Expert® software (Statease Corp, USA). The effects of individual parameters on esterase production was calculated by the following equation (Eqn.1)

$$\epsilon = (\sum_{\mu+} - \sum_{\mu-})/n \quad (1)$$

Where ε is the effect of parameter under study and "μ_+" and "μ_-"are responses (esterase activities) of trials at which the parameter was at its higher and lower levels respectively and "n" is the total number of trials. Analysis of variance (ANOVA) was performed on the data to determine the significance of fitted model and to test the significance of the effect of individual parameters on esterase production. The most significant parameters affecting esterase production were identified.

4.2.3.2. Optimization of significant parameters using response surface method

The significant parameters identified by the Placket and Burman design were optimized using a response surface methodology (RSM). Specifically a Box Behnken design (Box and Behnken, 1960) was used for this study where the effect of the significant variables was studied at three different levels. The design matrix with 17 experimental runs in a single block where the midpoint was replicated 5 times is shown in Table 4.3. The screened variables: inoculum concentration, medium pH and incubation time were coded as X_1, X_2 and X_3 respectively.

Table 4.3: Box Behnken design matrix for optimization of parameters identified by the fractional factorial design

Std order	Block	Inoculum (% v/v)		pH		Time (h)	
		Actual	Coded	Actual	Coded	Actual	Coded
1	1	2	-1	4.5	0	96	1
2	1	3	0	4.5	0	84	0
3	1	3	0	4.5	0	84	0
4	1	3	0	4.0	-1	72	-1
5	1	4	1	4.5	0	72	-1
6	1	3	0	4.5	0	84	0
7	1	4	1	4.5	0	96	1
8	1	2	-1	5.0	1	84	0
9	1	2	-1	4.5	0	72	-1
10	1	3	0	5.0	1	96	1
11	1	2	-1	4.0	-1	84	0
12	1	3	0	4.0	-1	96	1
13	1	4	1	4.0	-1	84	0
14	1	3	0	4.5	0	84	0
15	1	3	0	5.0	1	72	-1
16	1	4	1	5.0	1	84	0
17	1	3	0	4.5	0	84	0

The behavior of the system was modeled by a second order polynomial equation. The model equation used for the analysis is given below (Eqn. 2)

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} \beta_{ij} X_i X_j + \in \qquad (2)$$

Where, Y is the predicted response; β_0 is the offset term; β_i is the linear effect; β_{ii} is the squared effect, β_{ij} is the interaction effect, X_i and X_j are coded terms for independent variables under study and ε is the error factor.

For three variable systems the model equation is given below (Eqn. 3)

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_{23} + \beta_{23} X_2 X_3 + \in \qquad (3)$$

Regression analysis and estimation of the coefficients were performed using Design Expert ® v.8.01. Pareto chart of the effect estimates, the three dimensional response surfaces and/or contour plots were generated using or Design Expert® or Statistica® (Statsoft Inc, USA). The ideal levels and combinations of parameters were identified by optimization functions in the Design Expert software and experiments were run at these levels for validation of the model.

4.3. Results and discussion

4.3.1. Optimization of parameters affecting esterase production

Optimization of the parameters affecting esterase production by the isolate *Pseudozyma* was performed in two steps. The first step involved the screening of seven parameters considered to be important in esterase production by the isolate using a fractional factorial design (Plackett and Burman, 1946). The significant parameters identified by the factorial design were optimized using a response surface Box-Behnken design (Box and Behnken, 1960).

4.3.1.1. Screening of parameters influencing esterase production by Pseudozyma sp.

The effect of 7 parameters that were selected for the study was evaluated by conducting 8 runs of experiments that involved different combinations of the variables. The specified conditions of incubation were provided and the enzyme was extracted and assay carried out as outlined in the section 2.4 and 2.5.1 respectively

The Plackett and Burman experiments showed a wide variation in esterase production ranging from 141.7 U/ml to 2777.47 U/ml (Table 4.4). This variation shows the importance of optimizing these parameters for improving esterase production by the novel isolate.

Table 4.4. Plackett & Burman Experimental Design Matrix with the responses obtained

Std order	*Glucose (% w/v)*	*Peptone (% w/v)*	*Olive Oil (% v/v)*	*Inoculum Conc. (% v/v)*	*pH*	*Time (h)*	*$(NH_4)_2SO_4$ (% w/v)*	Esterase (U/ml)
1	0.5	0.6	0.1	2	6.5	120	0.4	169.29
2	1.0	0.6	0.1	1	4.0	120	1.0	1490.11
3	0.5	1.2	0.1	1	6.5	96	1.0	141.70
4	1.0	1.2	0.1	2	4.0	96	0.4	2685.52
5	0.5	0.6	0.3	2	4.0	96	1.0	2777.47
6	1.0	0.6	0.3	1	6.5	96	0.4	224.46
7	0.5	1.2	0.3	1	4.0	120	0.4	158.21
8	1.0	1.2	0.3	2	6.5	120	1.0	218.94

The effects of the individual parameters were found out by using the equation 3.

$$\epsilon = (\sum_{\mu+} - \sum_{\mu-})/n$$

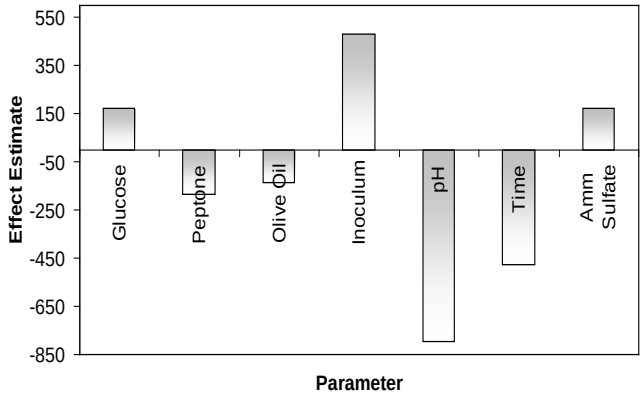
Where ε is the effect of parameter under study and "$\sum\mu_+$" and "$\sum\mu_-$"are sum of responses (esterase activities) at high and low levels of parameters of trials at which the parameter was at its higher and lower levels respectively and "n" is the total number of trials. The effects of the various parameters are given in Table 4.5.

Table 4.5. Estimated effects of individual parameters

Parameter	*Effect Estimate*
Glucose	171.55
Peptone	-182.12
Olive oil	-138.44
Inoculum	479.59
pH	-794.62
Time	-474.08
$(NH_4)_2SO_4$	173.84

pH, Inoculum concentration, and incubation time were noted to have the highest effects. While increase in inoculum concentration resulted in a higher esterase yield, either increase of pH or incubation time resulted in decrease of esterase yield. Increasing olive oil or peptone concentration was also found to have negative effects on enzyme production.

Fig.4.1. Pareto chart for the estimated effects of the selected parameters on esterase production.



Esterases being primary metabolites, the increase in yield of enzyme with increase in biomass (inoculum density) can be expected. Enzyme synthesis during fermentation is closely related to biomass concentration and morphology of the cells (Krastanov *et al.*, 2008). An increase in inoculum density allows the biomass density to increase rapidly (Bai and Zhao, 2012) and as a consequence, the expression of enzymes required in primary metabolism may increase. pH is another important parameter that can affect fermentative production of enzymes. The ideal temperature and pH for lipase production

by yeasts are extremely variable being related to the genus, species and even strains under study (Lock *et al.,* 2007). The way lipases behave under different pH is also highly different with the *Candida rugosa* lipase allowing a bulkier substrate at near neutral pH (pH 7.2) due to flap opening, while at acidic pH, it accommodates a more compact substrate due to the restricted flap opening (James *et al.,* 2003). These changes in properties of the lipase itself might affect the way the enzymes are induced. Increase in incubation time resulted in a decreased enzyme activity. Beyond 96h, esterase activity decreased and this could be due to the production of proteases by the culture. It may be noted that the culture was positive for protease (section 3.3.5)

The results were analyzed statistically and a first order polynomial equation (eqn.5) was derived to represent esterase production as a function of the independent variables

$$Y = 983.21 + 479.59X_4 - 794.62\ X_5 - 474.07\ X_6 \quad (5)$$

Y - Response (Esterase Activity)
X_4 - Type of Substrate
X_5 - Incubation temperature
X_6 - Incubation time

Adequacy of the model was tested and the parameters with significant effects were identified by the analysis of variance (ANOVA). The results of the ANOVA are given in Table 4.6. Model F value of 12.93 and the p value of 0.016 indicated that the model was significant and adequate to represent the system under study. The parameters with significant effects were inoculum concentration, pH of the medium and incubation time with confidence levels above 95% ($p \leq 0.05$).

Table 4.6. ANOVA (Analysis Of Variance) Table for the factorial model.

Source	*Sum of Squares*	*DF*	*Mean Square*	*F Value*	*Prob > F*
Model	8689352.56	3	2896450.85	12.933	0.0159
X_4	1840071.73	1	1840071.73	8.216	0.0456
X_5	5051303.99	1	5051303.99	22.554	0.0090
X_6	1797976.85	1	1797976.85	8.028	0.0472
Residual	895863.38	4	223965.84		
Corr. Total	9585215.94	7			

4.3.1.2. Optimization of critical parameters identified by factorial design

The three critical parameters that were found to have the maximum effect on the esterase production were taken up for further studies to optimize their levels using response surface methodology. The levels of glucose, peptone, olive oil and ammonium sulfate in the enzyme production medium were fixed at the levels that gave the maximum production of esterase (0.5%, 0.6%, 0.3%, 2%, and 1.0 % respectively) and the levels of the significant parameters identified by the factorial design ie – Inoculum concentration, pH and incubation time were optimized using a response surface Box Behnken design (Box Behnken, 1962). The Box Behnken experiment design and the experimental and predicted responses obtained for esterase production by *Pseudozyma* is shown in Table 4.7. The data was analyzed by multiple regression analysis and a second order polynomial equation was derived to represent the esterase production as a function of the independent variables tested.

$$Y= 6729 +1257X_1 + 2874X_2 - 322X_3 - 2637 X_1^{\ 2} - 1079 X_2^{\ 2} -1786 X_3^{\ 2} + 1182 X_1X_2 - 649 X_1X_3 + 431X_1X_2 \quad (6)$$

Where Y = predicted response (esterase yield), X_1, X_2 and X_3 are coded values of inoculum concentration, pH and Incubation time respectively.

Testing of the model was performed by the Fisher's statistical test for the analysis of variance (ANOVA) using Design Expert software and the results are shown in Table 4.8. ANOVA of the quadratic regression model suggests that the model is significant with a computed F value of 7.13 and a P>F of 0.0084. The value of multiple correlation coefficient (R) was 0.9495 indicating a better correlation. Table 4.8 also gives the P values of each of the parameters and their quadratic and interaction terms. The values of P>F less than 0.05 indicated that the model terms were significant and in this case X_1 X_2, $X_1^{\ 2}$ and $X_3^{\ 2}$ were found to be significant. There was no significant interaction between the parameters.

Response surface curve was plotted to understand the interaction effects of variables and for identifying the optimal levels of each parameter for attaining maximal esterase yield. Figure 4.2, 4.3 and 4.4 represent the response surfaces for the interaction effects of inoculum concentration and pH, inoculum concentration and incubation time, and pH and incubation time respectively

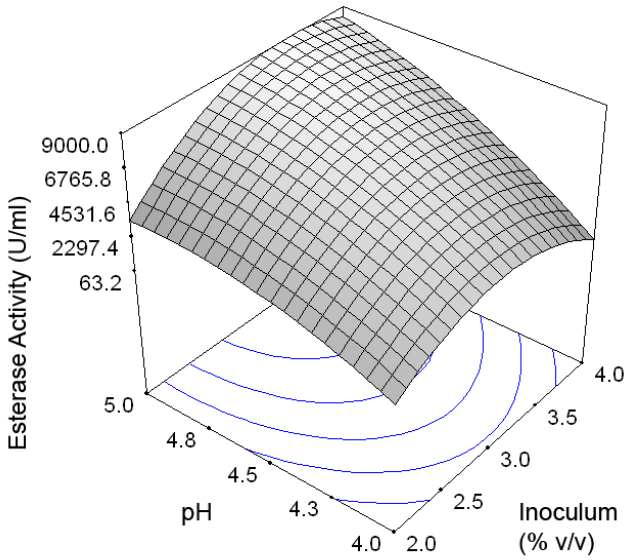
Table 4.7. Box Behnken design matrix and responses obtained for the response surface optimization of esterase production by *Pseudozyma sp.*

Std Order	Inoculum Conc. (% v/v)	pH	Incubation time (h)	Esterase Activity (U/ml)	
				Obtained	Predicted
1	2	4.0	84	418.85	63.18
2	4	4.0	84	763.68	213.32
3	2	5.0	84	2897.01	3447.37
4	4	5.0	84	7969.74	8325.41
5	2	4.5	72	1632.64	720.65
6	4	4.5	72	5250.77	4533.47
7	2	4.5	96	657.93	1375.23
8	4	4.5	96	1678.62	2590.61
9	3	4.0	72	474.02	1741.68
10	3	5.0	72	6267.18	6628.81
11	3	4.0	96	598.16	236.53
12	3	5.0	96	8113.33	6845.67
13	3	4.5	84	7600.50	6728.71
14	3	4.5	84	7805.64	6728.71
15	3	4.5	84	7651.80	6728.71
16	3	4.5	84	4472.31	6728.71
17	3	4.5	84	6113.33	6728.71

Table 4.8: ANOVA for quadratic model

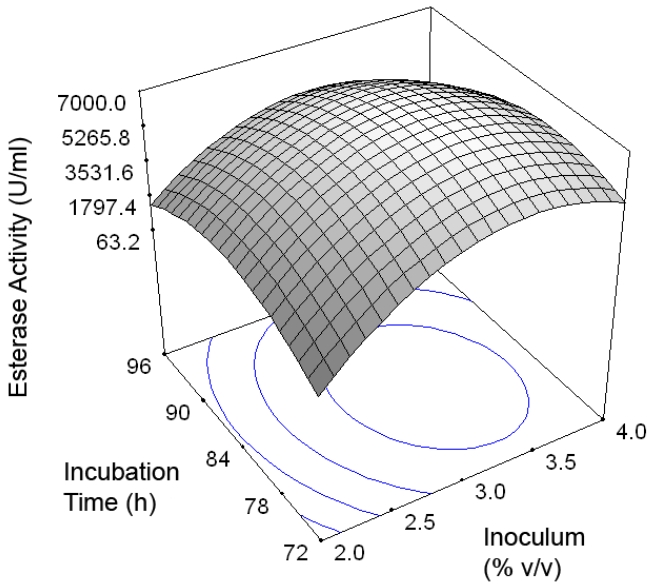
Source	*Sum of Squares*	*DF*	*Mean Square*	*F Value*	*Prob > F*
Model	139939088.43	9	15548787.60	7.13	0.0084
X_1	12641347.34	1	12641347.34	5.80	0.0470
X_2	66082169.44	1	66082169.44	30.30	0.0009
X_3	829839.12	1	829839.12	0.38	0.5569
X_1^2	29285450.90	1	29285450.90	13.43	0.0080
X_2^2	4903037.38	1	4903037.38	2.25	0.1775
X_3^2	13437289.52	1	13437289.52	6.16	0.0421
X_1X_2	5588259.60	1	5588259.60	2.56	0.1535
X_1X_3	1686673.64	1	1686673.64	0.77	0.4084
X_2X_3	741329.61	1	741329.61	0.34	0.5782
Residual	15268656.36	7	2181236.62		
Lack of Fit	7026731.68	3	2342243.89	1.14	0.4350
Pure Error	8241924.68	4	2060481.17		
Corr. Total	155207744.79	16			

Figure 4.2: Response surface plot showing the effect of inoculum concentration and pH on esterase production by *Pseudozyma* sp.



The highest esterase yields were obtained with an inoculum concentration of around 3.5 % and at a medium pH of 5.0. At the lower pH, maximal esterase activity was obtained at lower inoculum concentrations, while at pH 5.0, high enzyme activities were observed with increase in inoculum concentration. It may be speculated that at lower pH, the yeast cells are under stress resulting in a higher amount of enzyme production which could explain this phenomenon.

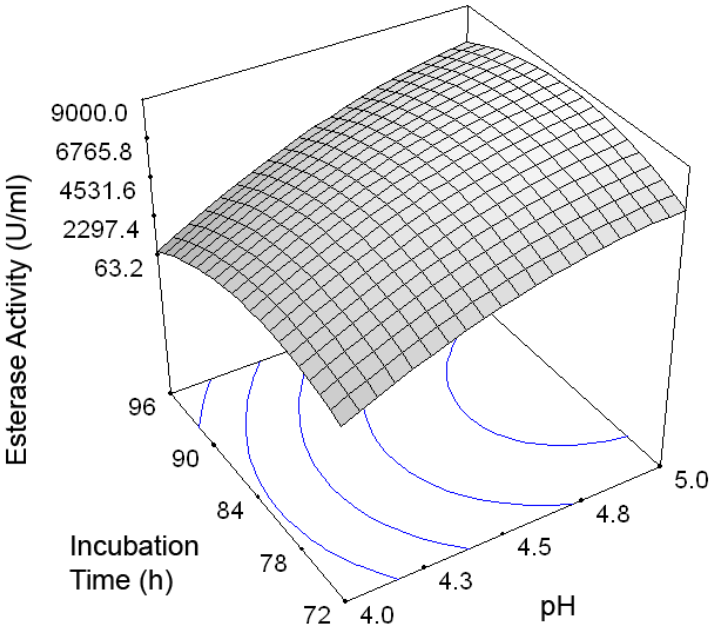
Figure 4.3: Response surface plot showing the effect of inoculum concentration and incubation time on esterase production by *Pseudozyma* sp.



Irrespective of the inoculum concentration, the maximal esterase yield was obtained at an incubation time of 80-84h (Fig 4.3). The inoculum concentration which gave maximal enzyme production was also almost independent of incubation time and was between 3 and 3.5 %.

The time required for obtaining maximal esterase yield was also influenced by the pH of the medium. At lower pH the esterase yield increased with increase in incubation time and peaked at around 78-80h while at higher pH, the peak production was observed between 80-84 h (Fig 4.4). However, it may be noted that there was not much difference in the incubation time needed for peak production and the interaction between these parameters were not so pronounced.

Figure 4.4 Response surface plot showing the effect of pH and incubation time on esterase production by *Pseudozyma* sp.



Optimization of the parameters (inoculum concentration, pH and incubation time) was done using the numerical optimization function in Design Expert®. The top five solutions suggested by the software are given in Table 4.9

Table 4.9: Conditions predicted for maximizing esterase production from *Pseudozyma sp NII 08165*

Number	*Inoculum (% v/v)*	*pH*	*Time (h)*	*Predicted esterase activity (U/ml)*
1	3.0	4.9	88	8279.6
2	3.8	5.0	81	8655.8
3	3.9	4.9	81	8445.9
4	3.2	4.8	84	8310.9
5	3.5	5.0	83	8975.5

Validation of the predicted conditions was done by performing the production study under optimized condition 5 which had the predicted maximal activity. Experiments performed under these conditions gave a yield of ~8300 U/ml which is comparable to the predicted value confirming the validity of the model. The improvement in production from the screening level production studies using the basal medium to the optimized conditions was 28 fold from 300 U/ml to 8300 U/ml.

4.4. Conclusion

Fermentative production of esterase from the novel strain Pseudozyma sp. NII 08165 was optimized following a two step approach involving an initial screening of parameters and identification of those which can significantly influence esterase production by the yeast. These parameters were then optimized using a Response surface –Box Behnken method. The optimization was highly successful and resulted in an overall improvement in yield of 28 times from 300 U/ml to 8300 U/ml. The yield is one of the highest lipase yields reported for yeast cultures indicating the potential for use of this culture for esterase production.

Chapter 5

Purification and characterization of esterase from *Pseudozyma*

5.1. Introduction

Protein purification is the essential first step for the characterization of function, structure and interactions of a protein of interest. Once a protein is purified, it is possible to study its enzymology, affinity for particular substrates, the structure function relationship etc. The purification strategies adopted to purify a desired protein, depends on the specific properties that make it distinctive from other proteins. Proteins can be purified based on its solubility, charge, size and binding affinity. Majority of the purification steps includes a precipitation/extraction step followed by a combination of chromatographic techniques. The most commonly reported purification strategies for lipases have ammonium sulphate precipitation followed by ion exchange, gel filtration or hydrophobic interaction chromatography (Saxena *et al.,* 2003; Dalal *et al.,* 2008; Chander and Shamsher, 2012). Two step purification strategies comprising of ammonium sulphate precipitation followed by hydrophobic interaction chromatography (HIC) was successfully employed by Saxena et al. (Saxena *et al.,* 2003) and Salameh and Wiegel (Salameh and Wiegel, 2007) for lipase purification from fungus and bacteria respectively. A similar purification strategy was adopted here for purifying one of the esterase isoforms of *Pseudozyma sp* NII 08165, designated as *LIP* 1 to homogeneity. Secreted isozyme profile of an enzyme depends to a major extent on the culture/fermentation conditions. Even though isozymes may be coded by the same gene family, they differ in their properties like isoelectric point, hydrophobicity, post translational modification like glycosylation etc (Trbojevic *et al.,* 2013). Hence, the culture conditions and the assortment of purification steps used, has a profound influence on the type of isoform that gets purified from a single preparation. Presence of inhibitory substances or existence of different isoforms together in the crude preparation may inhibit their biological activity. Purification of isoforms may thus improve its overall activity and stability (Trbojevic *et al.,* 2013). On the contrary, combined existence of different isozymes may impart interesting properties in the crude enzyme preparation. The studies done on the properties of the crude lipase from *Pseudozyma sp* indicated the great commercial potential for this enzyme, especially

where purity would not be of major concern. So the extent of enzyme purity required thus depends on the intended end use of the protein.

Lipases/Esterases with novel properties are always in demand due to the large number of synthetic reactions which these enzymes can possibly catalyze and for which enzymatic routes are currently not available (Cardenas et al, 2001). Novel specificities, tolerance to extremes of pH, temperature and/or salt tolerance are features often desired in lipases. Biocatalysis in organic solvents are advantageous due to several reasons including the ability to perform reactions restricted kinetically or thermodynamically in water, increased solubility of certain substrates in organic solvents, suppression of undesirable side reactions, possibility to control or modify the enzyme selectivity, increased thermal stability in organic solvents etc (Zaks and Klibanov, 1988; Hernandez-Rodriguez *et al.,* 2009; Dmitrijevic *et al.,* 2011). Among the high number of lipases described in literature, only a few belonging to a few species have been demonstrated to have the adequate stability and biosynthetic activity capabilities to allow routine use in organic solvents (Eltaweel *et al.,* 2005). It has been observed that the enzyme activity and stability is higher in hydrophobic solvents compared to hydrophilic solvents since the latter can strip the water required for activity (Zaks and Klibanov, 1988). In general hydrophilic solvents can strip water from an enzyme's surface and penetrate more easily into the active site than the hydrophobic solvents (Gorman and Dordick, 1992). This can cause denaturation or inactivation and there are not many rational strategies for enhancing the stability of enzymes in hydrophilic solvents (Park *et al.,* 2012). At the same time, there are reactions which perform better in polar organic solvents and hence the lipases/esterases that work in such systems are also highly desired in the industry (Dmitrijevic *et al.,* 2011). The *Pseudozyma* sp NII 08165 isolated at the biotechnology division of CSIR-NIIST was found to produce esterase activity which was sustained at higher temperature and under high salt concentration. The enzyme was more active under alkaline pH and it retained activity in presence of polar organic solvents like ethanol and methanol. The present work explored the possibility of using this novel isolate as a source of lipase active in polar organic solvents and for characterizing the enzyme for properties that are desirable for industrial lipases.

5.2. Materials and methods

5.2.1. Studies on the properties of the partially purified esterase

5.2.1.1. Partial purification of Pseudozyma esterase

Lipase production was done by submerged fermentation as described in section 2.4. Crude enzyme preparation was concentrated by ultra filtration using a 10kD cut off membrane and was subjected to Ammonium sulfate precipitation using 60 % $(NH_4)_2SO_4$. The pellets were dissolved in a minimal volume of 0.1 M Tris buffer (pH 8.0) and were dialyzed against excess volume of 0.02 M Tris buffer (pH 8.0) for salt removal. The concentrated enzyme sample as above was used for studies on the properties of the lipase. The enzyme was tested for tolerance to temperature, solvents, salt, pH and its stability at elevated temperatures.

5.2.1.2. Temperature tolerance and Thermostability

The temperature tolerance of lipase was studied by assaying the enzyme samples at 37, 50, 60 and 70 ^{o}C. The activities obtained were represented as percentage of activity in comparison to the control enzyme assayed at 37 °C. Temperature stability of the enzyme was studied by incubating the enzyme at different temperatures (40, 50, 60 and 70 °C) for 1h followed by assay of the enzyme. The activities obtained were compared to the control enzyme incubated at room temp (30 ± 2 ^{o}C) and assayed at 37 ^{o}C.

5.2.1.3. pH tolerance and pH stability

pH tolerance of lipase was evaluated by performing the assays in different buffers (Na-acetate – pH range 2-5, Na-Phosphate buffers – pH range 6-8, Tris buffer (pH range 8-10).The activity and stability at different pH was tested by incubating the enzyme in different buffers for 1h and then performing the assays in the same buffer at 37 ^{o}C.

5.2.1.4. Solvent Tolerance

The enzyme was assayed in presence of 25 % and 50 % of the water miscible organic solvents – isopropanol, acetonitrile, ethanol or methanol. The solvents were incorporated into the assay mixture so as to get the required final concentration and the assays were performed at 37 $^{\circ}$C.

5.2.1.5. Halo Tolerance

The salt tolerance of the enzyme preparation was tested by performing the lipase assays in presence of varying concentrations of sodium chloride. NaCl was added to the assay mixture to obtain final concentration in the range 50mM -2M and assay was performed.

5.2.2. *Purification of the LIP*1 *esterase of Pseudozyma sp NII 08165*

5.2.2.1. Ultra filtration

Lipase production was carried out under optimized media and culture conditions in shake flasks. Cell free supernatant (1950 ml) obtained after centrifugation of cell homogenate at 4°C and 10,844 G for 15 minutes, was concentrated by ultra-filtration using a 50kD cut off membrane.

5.2.2.2. Ammonium sulfate precipitation

Retentate obtained after ultra filtration (300ml) was further purified by ammonium sulphate fractionation. The fraction obtained from the 60% $(NH_4)_2SO_4$ precipitation (17.5ml) showing maximum lipase activity was dialyzed against 20 mM HEPES buffer (pH 8.0) containing 1.0 M $(NH_4)_2SO_4$.

5.2.2.3. Hydrophobic interaction chromatography (HIC).

The dialyzed sample after ammonium sulphate precipitation was directly loaded onto Butyl Sepharose column (1.5 × 30 cm) pre-equilibrated with the same buffer. Elution of bound proteins was done with a 1.0 – 0.0 M linear gradient of $(NH_4)_2SO_4$ in 0.02 M HEPES buffer (pH 8.0) at a flow rate of 1ml min^{-1}. Fractions of 2ml were collected and assayed for lipase activity. The active fractions were pooled and dialyzed against 0.02 M HEPES buffer to remove salt.

5.2.3. Enzyme Characterization

5.2.3.1. Molecular weight determination

The HIC purified enzyme was analyzed in Native PAGE and checked for purity and activity of the protein. The purified protein was then de-glycosylated to obtain the correct molecular weight of the protein. De-glycosylation was conducted with 1-20µg purified protein. The sample was pretreated at 95 °C for 5 minutes with denaturing buffer. A 25 µl reaction was set up with treated protein, reaction buffer (1X) and Endo H_f enzyme (NEB, USA) according to manufacturer's protocols. The deglycosylated protein was then run on SDS PAGE. The gel was silver stained as described in section 2.6.1. Molecular weight was determined according to Laemmli (Laemmli, 1970).

5.2.3.2. Enzyme kinetics

Enzyme kinetic studies were performed and the Michaelis–Menten curve fitting and the determination of *V*max and *K*m were performed using GraphPad Prism® software (GraphPad Software Inc. USA). The kinetic parameters of the purified lipase were determined at the optimum temperature of 60 °C and pH 8.0, using 0.05-0.7 M pNPP as substrate.

5.2.3.3. Properties of purified isoform LIP 1

The purified lipase isoform was analyzed for its solvent, temperature and salt tolerance as described in section 5.2.1.

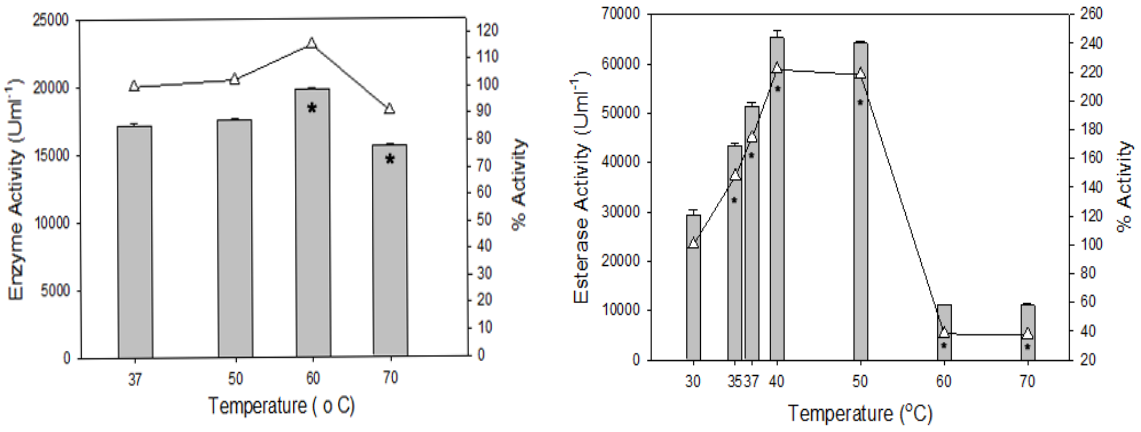
5.3. Results and discussion

5.3.1. Studies on the properties of partially purified enzyme

5.3.1.1. Temperature tolerance and temperature stability

Thermostable lipases are always in demand because of the high melting point of the several lipid substrates and also due to the possibility of achieving higher reaction rates (Janssen *et al.*, 1994; Sangeetha *et al.*, 2011). The partially purified esterase from *Pseudozyma* sp NII 08165 showed increased activity at 60°C (17134 to 19770 Uml^{-1}) and > 90 % of the activity was retained at 70°C (15550 Uml^{-1}) which may qualify it as a thermo-tolerant lipase (Fig 5.1A). Pre-incubation of enzyme at temperatures ranging from 35-50 °C actually increased the activity of the enzyme preparation in comparison to the control (pre-incubated at 30 °C). Incubation at either 40 °C or 50 °C resulted in ~ 220 % increase in enzyme activity indicating the stability of the enzyme at these temperatures. Nevertheless, the enzyme lost its activity significantly after 1h incubation at either 60 °C or 70 °C (Fig 5.1B). In the latter case the activity retained was ~40 % of the control. .

Fig 5.1: Temperature tolerance and stability of partially purified esterase preparation from *Pseudozyma* sp. NII 08165



A: Temperature tolerance

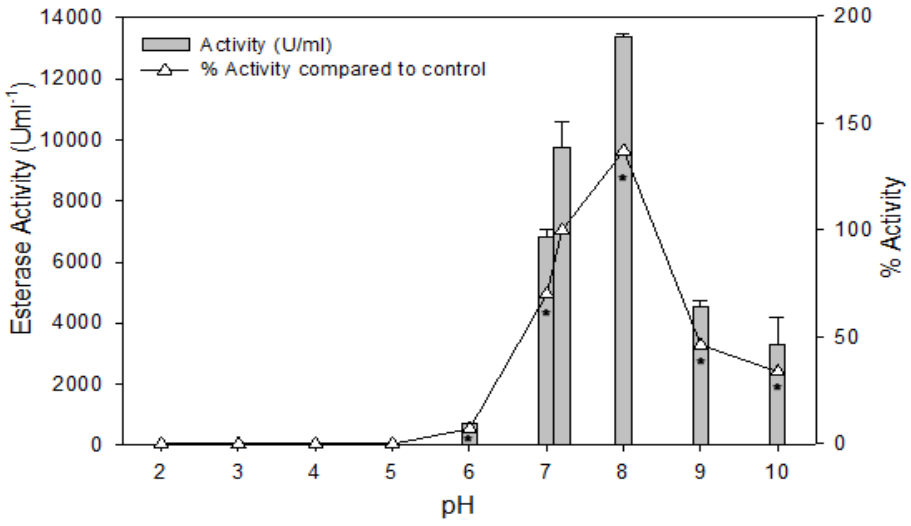
B: Temperature Stability

Bars represent enzyme activity and open triangles shows the % activity retention compared to control
** Enzyme activity values significantly different from control*

5.3.1.2. pH tolerance and pH stability

Neutral to slightly alkaline pH supported maximal esterase activity for the *Pseudozyma* enzyme. Maximal activity was obtained at pH 8.0 which was ~140 % higher than the control (pH 7.2). While the enzyme retained 70 % of its activity at neutral pH, the activity retention for pH 9.0 and 10.0 were only 46% and 34%. The enzyme was not active in the acidic pH, indicating a confined operating range. The optimal pH range for activity was 7-8, with pH 8.0 supporting maximal activity (Fig 5.2). However, the ability of the enzyme to act at pH 9.0 and 10.0, positions itself with a definite advantage for use in applications that require alkaline conditions.

Fig 5.2: Activity of the partially purified *Pseudozyma* esterase at different pH



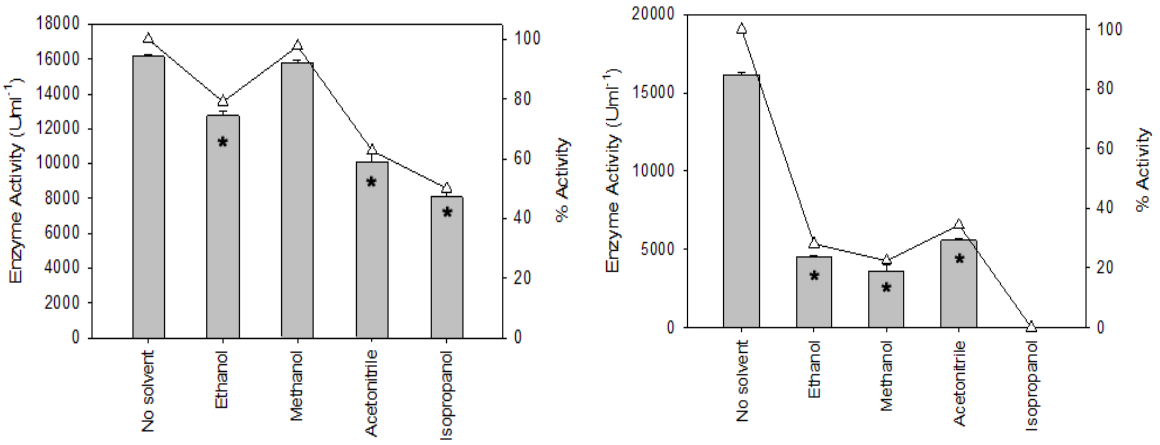
** Enzyme activity values significantly different from control*

5.3.1.3. Solvent tolerance

Lipases/Esterases are more susceptible to inactivation in presence of water miscible organic solvents, and tolerance to polar organic solvents is a highly desired property in lipases due to a range of reactions requiring such solvents (Hernández-Rodríguez *et al.*, 2009; Doukyu and Ogino, 2010). The *Pseudozyma* lipase was active in 25% concentration of all the tested organic solvents. In 25 % methanol, 97% of the activity was retained while 80% of activity was retained in presence of the same

concentration of ethanol (Fig 5.3A). The tolerance to acetonitrile and iso-propanol was 63% and 50 % respectively. Though there are reports of lipases with enhanced activity or activity retention in polar organic solvents (Kakugawa *et al.,* 2002; Karadzic *et al.,* 2006), the *Pseudozyma* lipase differed from them in having high enzyme activity. At 50 % solvent concentration, the enzyme lost most of its activity in all the tested solvents. The maximum activity retention at this concentration was 34 % for acetonitrile (Fig 5.3B). The enzyme completely lost its activity in 50 % iso-propanol while 75-80 % activity was lost in ethanol and methanol respectively. Nevertheless, it may be observed that the enzyme is active in presence of 25 % of the major polar organic solvents used in synthesis.

Fig 5.3: Solvent tolerance of partially purified esterase from *Pseudozyma* sp. NII 08165



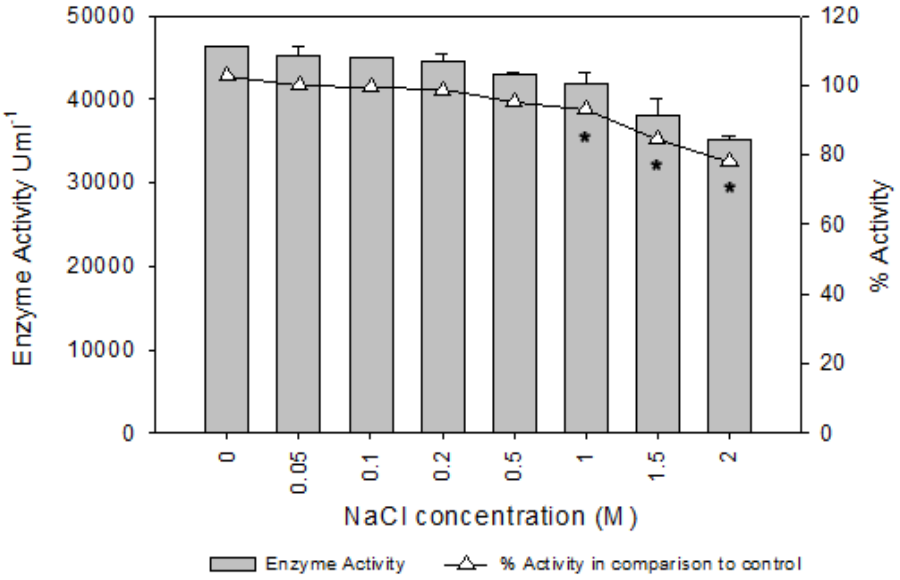
A: 25 % Solvent *B: 50 % Solvent*

Bars represent enzyme activity and open triangles shows the % activity retention compared to control.
** Enzyme activity values significantly different from control*

5.3.1.4. Halo tolerance

The lipase from *Pseudozyma* sp seemed to be halo tolerant as it was active in buffer containing 0.05-2 M NaCl and it retained >80% of activity at 2.0 M NaCl concentration. (Fig 5:4). While lipases from halophilic microbes are mostly active only at high salt concentrations, the salt tolerant lipases from mesophiles can act both in the absence and in a range of NaCl concentrations. The *Pseudozyma* lipase may be considered under the latter category which might be advantageous in several applications like waste water and oil spill management (Pandey *et al.,* 1999; Frutos *et al.,* 2002).

Fig 5.4: Halo-tolerance of partially purified *Pseudozyma* esterase



** Enzyme activity values significantly different from control*

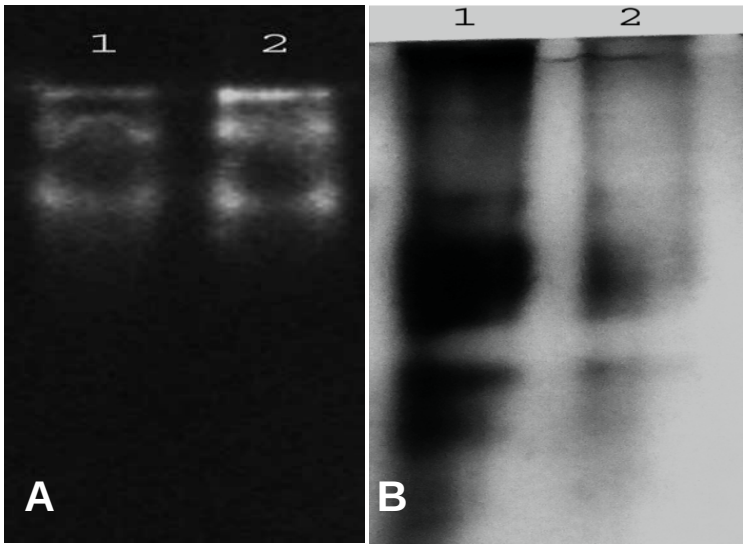
5.3.2. Purification of LIP 1 enzyme

Crude esterase was concentrated and fractionated by ultra filtration through a 50 kD cut off membrane to obtain an 8.5 fold purification, followed by ammonium sulphate fractionation. Maximal lipase activity was detected in the 60% (w/v) ammonium sulphate fraction, though the other fractions also contained appreciable amount of lipase activity. Activity staining of the partially purified esterase using 4-methyl umbelliferyl palmitate (Roberts, 1985) indicated the presence of multiple isoforms of lipase (Fig 5.5A & B). The enzyme was further purified to ~ 82 fold through hydrophobic interaction chromatography. The specific activities of lipase was 45, 383, 1184 and 3694 Umg^{-1} respectively for the crude, 50kD retentate, 60 % $(NH_4)_2SO_4$ fraction and HIC fractions (Table 5.1). The purified protein which formed a single band in SDS PAGE was designated as esterase *LIP1*.

Table 5.1: Summary of purification of esterase from *Pseudozyma* sp NII 08165

	Volume (ml)	Esterase activity (U)	Total activity (U)	Protein content (mg ml^{-1})	Total protein (mg)	Specific activity (Umg^{-1})	Fold purification
Crude enzyme	1950	100	195390	2.2	4290	45	100
Ultra filtration (50kD) Retentate	160	1203	192483	3.14	502	383	851
60% $(NH_4)_2SO_4$ fraction	17.5	3179	55638	2.7	47	1184	2631
HIC fraction	40	314	12560	0.085	3.4	3694	8208

Fig. 5.5: Zymogram analysis of crude and partially purified esterase from *Pseudozyma* sp. NII 08165



A) Zymogram analysis of Pseudozyma esterases. Lanes: 1- Crude enzyme, 2-Ammonium sulfate fraction.

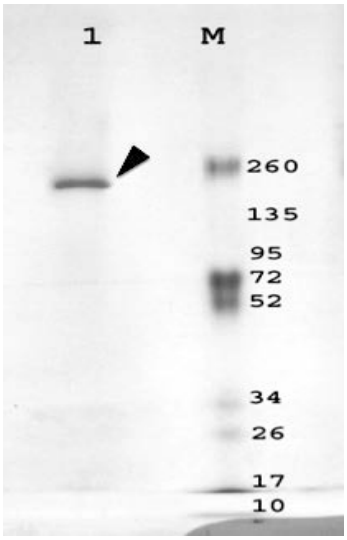
B) Native PAGE analysis of esterases. Lanes: 1 Crude enzyme, 2-Ammonium sulphate fraction (Protein visualization by silver staining.

5.3.3. Characterization and Properties of LIP1 from Pseudozyma SP NII 08165

5.3.3.1. Molecular weight determination

The purified lipase on SDS-PAGE and silver staining showed a single band of molecular weight 175 kDa (Fig 5.6).

Fig. 5.6: SDS PAGE analysis of purified *LIP*1 from *Pseudozyma* sp NII 08165



Lane 1 – Purified LIP1 esterase

Lane M- SDS broad range protein ladder (Spectra ®, Thermo Scientific)

The 175 kD LIP1 esterase protein band is indicated by arrow head

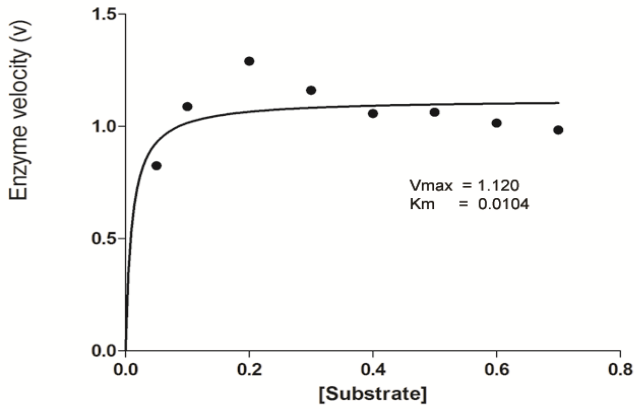
5.3.3.2. Enzyme kinetics

Kinetic parameters of the purified lipase *LIP* 1, was determined in a normal assay mixture with pH 8.0, incubation temperature of 60 °C and using a pNPP concentration range of 0.05-0.7 mM (Table 5.2). Michaelis–Menten kinetics was fitted and the *K*m and *V*max was determined to be 0.01mM and 1.12 mM min^{-1} (Fig.5.7)

Table 5:2 Substrate concentrations [S] used for kinetics study and obtained velocities (V)

[S]	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7
V	0.82	1.08	1.29	1.16	1.06	1.06	1.01	0.98

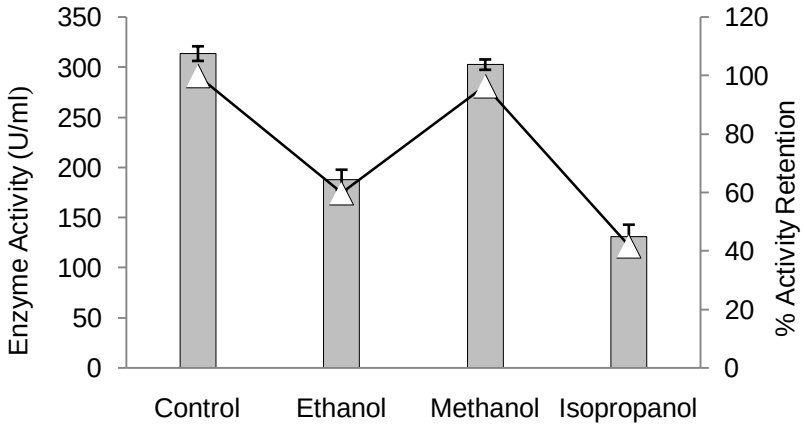
Fig 5.7: Michaelis Menten Curve for *Pseudozyma* sp. *LIP1*



5.3.3.3. Solvent tolerance of LIP1

The purified *LIP* 1 showed only 60% and 42 % activity retention in ethanol and iso-propanol whereas the crude lipase had shown 80% and 50% activity retention respectively in these polar organic solvents. It may be noted that there were more than one lipase isoform in the crude enzyme and the isoforms other than *LIP*1 could have contributed to its higher solvent tolerance. Interestingly, the activity retention for purified *LIP*1 in 25% methanol (97%) was identical to the crude enzyme Fig 5.8.

Fig 5.8: Solvent tolerance of purified *LIP* 1



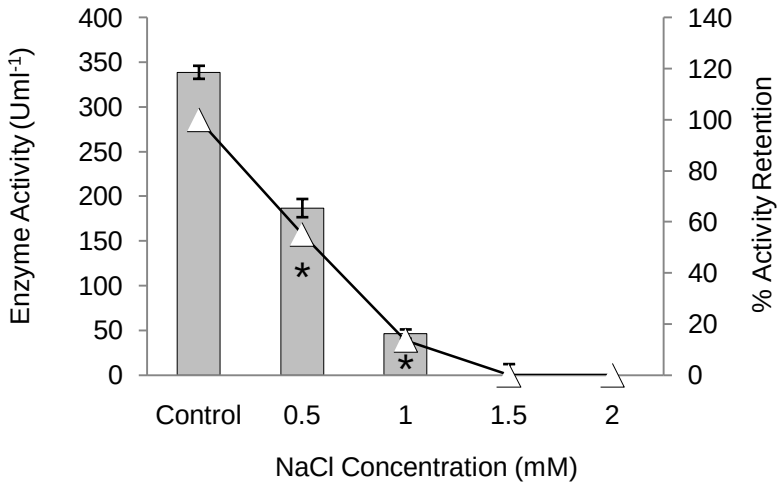
Being a polar organic solvent, methanol is the most common acyl acceptor used in the transesterification reaction for biodiesel production. Lipases stable in polar organic solvents are advantageous since the water miscible solvent and water can act together as a homogenous co-solvent system and possibly help in reactions involving substrates that

are otherwise insoluble in water (Park *et al.*, 2012). It was also reported that water miscible organic solvent systems enhanced hydrolysis of hydrophobic substrates compared to the hydrophilic substrates and such solvents systems may help to modulate the lipase activity (Tsuzuki *et al.,* 2003). There are several efforts worldwide to enhance the stability and activity of lipases in polar organic solvents ranging from prior treatment with such solvents (Chamorro *et al.*, 2001) to structural modifications of the protein (Park *et al.*, 2012). A lipase naturally tolerant to polar organic solvent assumes importance in this context and the *Pseudozyma* sp lipase with its added advantage of high enzyme titres makes it a potent candidate for industrial applications both in hydrolysis and synthesis.

5.3.3.4. Halo tolerance of purified LIP 1

The halo-tolerance of purified lipase decreased sharply compared to the crude enzyme (Fig 5.9). The partially purified lipase which showed more than 80% activity retention in 1.5- 2.0 M NaCl (Fig. 5.4) showed complete absence of activity at 1.5 M salt concentration indicating that the halo-tolerance exhibited by the crude enzyme was contributed by the isoforms other than *LIP*1.

Fig 5.9: Halo tolerance of purified *LIP* 1

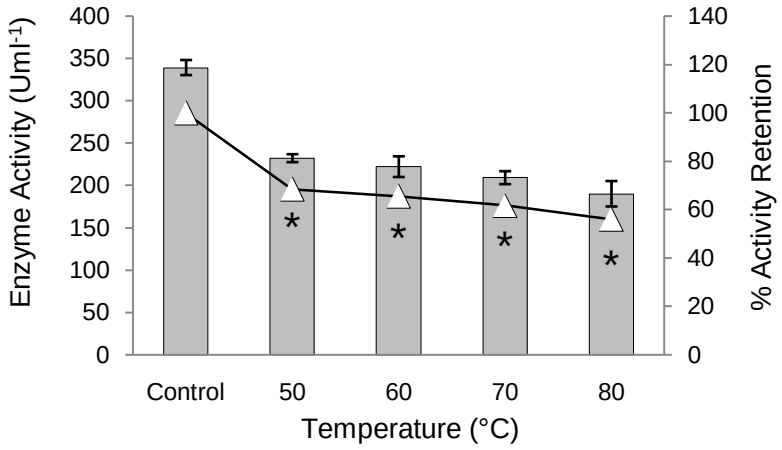


5.3.3.5. Thermo tolerance

The temperature tolerance for purified *LIP* 1 also decreased significantly compared to the crude enzyme. While the partially purified lipase retained 90% activity after treating the

enzyme at 70°C for 1 hr, purified enzyme could retain only 62% of its original activity under the same conditions (Fig 5.10).

Fig 5.10: Thermo tolerance of purified *LIP* 1



5.4. Conclusion

The studies done on the properties of the partially purified esterase from *Pseudozyma* sp NII 08165 indicated the great potential of this enzyme. The partially purified esterase was active at 60 °C and had an enhanced activity after incubating at 50 °C for 1h. The enzyme may therefore be called thermo tolerant. Besides temperature tolerance, the enzyme preparation also exhibited other interesting features like an alkaline pH range for optimal activity, tolerance to water miscible organic solvents and the ability to act in a wide range of salt concentrations. While there could be enzymes which have better performance in any of these aspects, the occurrence of all these properties in a single enzyme preparation is interesting.

The *LIP*1 esterase from *Pseudozyma* sp NII 08165 was purified to homogeneity and the purified isoform retained 97% of its activity in 25% methanol -a polar organic solvent which is the most common acyl acceptor used in the transesterification reaction for biodiesel production. Esterases tolerant to polar organic solvents have several important applications and a naturally solvent tolerant enzyme like the *Pseudozyma* esterase will be highly useful in synthesis and hydrolysis applications. Even though the partially purified esterase preparation exhibited temperature and halo tolerance, these properties were not observed in the purified *LIP*1 indicating that those properties might have been contributed by other esterases in the preparation.

Chapter 6

Construction and screening of metagenomic libraries for novel lipases

6.1. Introduction

Though a large number of microbial lipases have been studied for their applications as well as for understanding their basic biochemistry, only a few of them are actually used in the industrial applications (Cardenas *et al.*, 2001). Many of them have been cloned and in-depth studies have been performed on the properties of these enzymes. The specificities required for each industrial application are different as are the properties of the enzyme suited for those. Newer activities and better performance and properties for the enzyme are in constant requirement. Enzyme manufacturers now offer kits with different lipases against which the desired reaction may be screened. Also tremendous improvements have been made in understanding lipase function and in enzyme modifications for improving activity or imparting desired features.

Despite the commercialization of a large number of lipases from varying sources, there still exists a demand for novel lipases due to the tremendous amount of unique bio-catalytic possibilities where these enzymes can be employed. Lipases with thermo-tolerance are used in the hydrolysis of hard fats while halo tolerant lipases and lipases with different fatty acid chain length specificities are desired for biotransformation reactions. Similarly, solvent tolerant enzymes are highly in demand for organic synthesis. Lipases stable under extreme conditions are also useful in waste water and oil spill treatments (Hasan *et al.*, 2006; Marhuenda-Egea and Boneta, 2002). Indeed lipases have been screened for and produced from a variety of microorganisms (Kojima *et al.*, 2003, Castro-Ochoa *et al.*, 2005, Fickers *et al.*, 2006). Access to a wider range of lipases would help to address several catalytic reactions for which currently enzymatic routes are not available or not economically feasible (Clausen, 1997).

Most of the commercial lipases are extra-cellular enzymes of microbial origin, allowing for the inexpensive manufacture of crude enzyme extracts. Such preparations, however, can vary from lot-to-lot, due to the production of different isoforms of the enzyme by the source organism in varying amounts depending on cultivation conditions (Weber *et al.*, 1995), Genetic engineering techniques facilitate the economic production of pure enzymes and the preparation of tailor-made enzymes for specific applications.

The genes of many lipases have been cloned, and over-expression of lipases in suitable hosts has been achieved (Schmidt-Dannert *et al.,* 1998, Vakhlu and Kaur, 2006). Cloning of lipase genes could be thought as the most appropriate approach for production of the pure lipase due to the existence of several isoforms of the enzyme in many organisms.

The classical approach to isolate novel and potential lipases is to screen a wide variety of microbes for the desired type of activity or features. The enzymes and then the corresponding genes are then isolated from the identified organisms. However, in such methods the major fraction of microbial diversity in the environment is lost due to the inability to grow a huge fraction of the microbes in pure culture (Henne *et al.,* 2000). It has been estimated that 99% of the microorganisms in nature cannot be cultivated using standard techniques (Amman *et al.,* 1995). Many approaches currently used to explore the diversity and the potential of microbial communities are biased because of the limitations of cultivation methods (Caldwell et al, 1997). Classical cultivation techniques and media do not satisfy the growth requirements of several microbes.

Alternative approach is to use DNA based methods that do not require cultivation of the microorganism (Henne *et al.,* 2000). DNA can be isolated from environmental samples without culturing the organisms. This DNA can serve as templates for PCR amplification of the desired genes. However this method is also limited since the design of primers is typically based on the conserved sequences of similar products as the one in question. This method thus would not yield novel products. Another way is to use the environmental DNA for construction of DNA libraries, and to clone directly the functional genes from environmental samples. This method has the advantage that information on the sequences is not needed prior to cloning. Also the libraries thus prepared can be used to screen for other protein products of interest. This methodology of constructing metagenomic libraries is the most versatile of all methods designed to study and utilize the physiology and genetics of uncultured microorganisms. Once the genomic DNA from the environment is isolated and cloned into a culturable microorganism, the genetic information is captured as a library which can be studied and preserved (Handelsman, 2004). The analysis/screening of the library can be sequence based or functional. The former method involves the sequencing of entire clones or those of interest, whilst the latter involves screening the clones for the desired function. The success of the latter method depends on the efficiency of the transcription and translation of the environmental DNA sourced genes in the culturable host. Both of these methods

have been successfully employed to screen metagenomic libraries and to isolate novel genes (Handelsman, 2004).

Prospecting for novel lipases through metagenomics have been widespread due to the ever increasing demand for novel lipolytic activities useful in synthetic and biotransformation reactions apart from lipid hydrolysis. Many authors have successfully isolated novel lipase genes which otherwise would not have been discovered (Henne *et al.,* 2000; Rhee *et al.,* 2005). The success of metagenomics relies on the efficiency of the main steps which includes the isolation and manipulation of genetic material, library construction and the analysis and screening of genetic material in the metagenomic library.

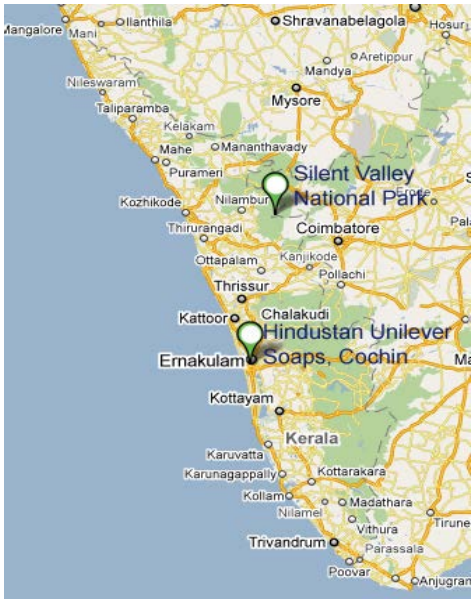
In this study soil samples from two unique sources - unexplored virgin forests of Silent Valley National Park (Kerala, India) as well as effluent sludge from a soap and detergent factory were used. The latter was expected to be an enriched source of micro-flora possessing lipolytic activity for construction of metagenomic libraries. Earlier attempts for isolating novel lipolytic genes from activated sludge and waste water treatment plants have been successful (Zhou *et al.,* 1996; Glogauer *et al.,* 2011). Esterases with important properties like thermo tolerance and solvent tolerance were isolated from the effluent sludge metagenome. The occurrence of relatively high proportion of esterase positive clones from the sludge sample indicated the suitability of this sample for isolation of novel lipase activities. Crude enzyme samples prepared from the clones were studied for their biochemical properties. Esterases isolated from some of the positive metagenomic clones possessed multiple important properties like thermo, halo and solvent tolerance.

6.2. Materials and methods

6.2.1. Collection of environmental soil samples

Soil samples were collected from virgin forests of Silent Valley National Park spanning a sampling coverage of ~ 50 km^2. Sludge samples were collected from different locations along the effluent outlet of Hindustan Unilever Soaps, Cochin, a soaps and detergent factory at Ernakulam, Kerala, India. The samples were transported to the lab under sterile conditions and DNA extractions were performed immediately. Forest soil samples and the sludge samples were pooled separately before DNA isolation

Fig 6:1 shows the different locales of sample collection.



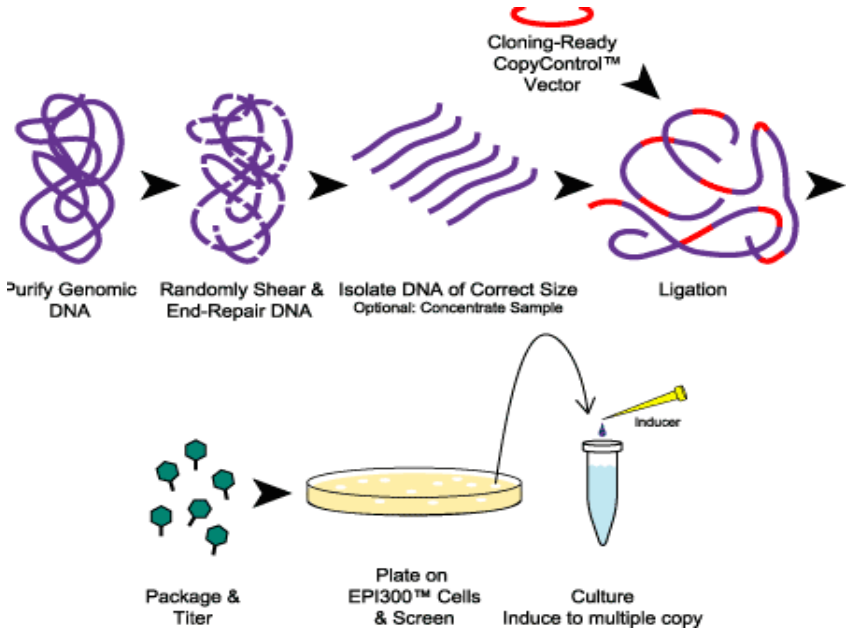
6.2.2. Isolation and purification of environmental DNA

DNA was isolated from 5g of pooled sludge samples using SDS-CTAB method (Zhou *et al.*, 1996) and was purified using silica beads for removing the humic content. DNA bound to the silica beads in presence of high chaotropic salts were repeatedly washed with 80% ethanol and were allowed to settle. The pellet was air dried and DNA was dissolved in nuclease free water. The DNA purity and concentration were analyzed by measuring UV adsorption using a Nanodrop ND-1000 spectrophotometer and by agarose gel electrophoresis. Alternatively, DNA was isolated using a commercial kit from MoBIO (Power Max soil DNA extraction kit) following the manufacturer's protocols.

6.2.3. Construction of metagenomic libraries: Fosmid Library

Fosmid libraries were constructed using the Epicentre® fosmid library construction kit. The purified genomic DNA was end repaired and size separated by pulse field gel electrophoresis in a 1.2 % agarose gel in 0.5 X Tris Borate-EDTA buffer, at 4 °C and 6V for 30 h in a Biorad® Chef Mapper III PFGE unit. Fragments of 35–45 kb were cut from the gel. The gel was digested with agarase, and the DNA was extracted by a single precipitation step with isopropanol and washed with 70% ethanol. The purified DNA was ligated to the CopyControl™ pCC1FOS fosmid vector (Epicentre, USA), and packaged into phages that were used to infect T1 resistant EPI300-T1 *Escherichia coli* cells (Fig 6.2). The bacteria were either plated on 12µg/ml chloramphenicol containing plates and grown overnight at 37 °C, or stored at -70 °C before plating in liquid selective media. The infected cells were frozen in 20% glycerol at –80 °C for long-time storage.

Fig 6:3 Outline of fosmid library construction



6.2.3.1. Confirmation of fosmid clones

Fosmid library was confirmed by induction. Overnight grown clones when treated with fosmid auto induction solution which cause subsequent amplification of the fosmids to high copy number. Fosmids were then isolated using standard plasmid preparation protocols as described in section 2.8. Phenol: Chloroform step was excluded to reduce shearing of vectors possibly harboring large inserts.

6.2.3.2. Insert size determination of fosmid clones

Fosmids from randomly selected clones were subjected to complete digestion with *Hind* III for 1h. Agarose gel electrophoresis was done to analyze the digested fragments. Fermentas 1 kb plus DNA ladder® (Fermentas, USA) was used as the marker. The average insert size of the clones was determined by comparing the size of the digested fragments with the standard size markers.

6.2.3.3. Screening of fosmid clones for esterases

Fosmid clones were screened for esterases in LB-tributyrin plates as outlined in Sussane et al (Susanne *et al.*, 1999). The plates were prepared by adding 1.5 ml of an emulsion of 50 % (v/v) tributyrin and 5% (w/v) gum arabic into 100ml of molten sterile LB agar. The emulsion was prepared by sonication of tributyrin and gum arabic solutions for 3 min at room temperature (30 ± 2 °C). The clones were spotted on the agar plates using sterile toothpicks and were incubated for 96 h at 37 °C.

6.2.4. Construction of metagenomic libraries: BAC library

BAC library was constructed in pBeloBAC® vector (NEB, USA) with soil DNA isolated from Silent Valley soil samples as well as effluent sludge samples. The DNA was purified using MoBio Power Max® soil DNA extraction kit. The purified DNA was end repaired, ligated with BAC vector and transformed into electro competent *E.coli* DH10B cells produced as outlined under section 2.9.

6.2.4.1. Confirmation of BAC clones

Plasmids were isolated from randomly selected clones as described in section 2.8 and agarose gel electrophoresis was performed to observe the position shift compared to control vectors.

6.2.4.2. Insert size determination of BAC clones.

Plasmids from randomly selected BAC clones were isolated as described in section 2.8 and restriction digestion was performed with *Hind* III for 1 hour at 37°C. The digested samples were run in an agarose gel (section 2.6.2). Average insert size of the clones can be calculated from the gel using the size of digested fragments compared to the marker (1 kb plus DNA ladder).

6.2.4.3. Screening of BAC clones for esterases

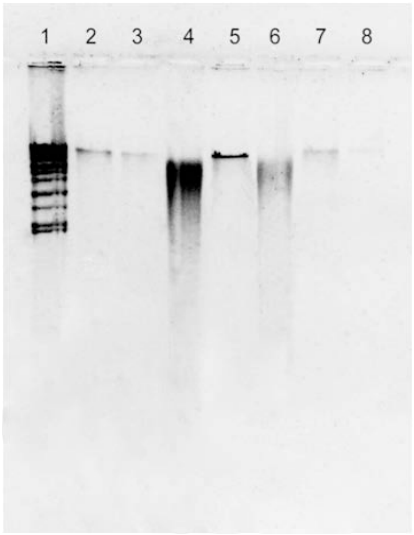
BAC clones were screened for esterases in LB-tributyrin plates as described above in section 6.2.2.1.3.

6.3. Results and Discussion.

6.3.1. Isolation and purification of metagenomic DNA

Yield of DNA from the effluent sludge was high when the commercial Soil DNA extraction kit was used for isolation. The method yielded 35µg of DNA/g of effluent sludge whereas only 25µg /g sludge was obtained with the SDS-CTAB method. Recovery of DNA after silica bead purification of the latter sample was 9µg. Purity of the DNA as determined by λ260/280 absorption ratio was 1.88 for the DNA isolated using commercial kit and 1.09 for SDS-CTAB method after silica bead purification. However, the size of DNA isolated using the SDS-CTAB method (~35kb) was higher than that obtained using the commercial kit (~25kb) (Fig 6.3)

Fig 6.3: Agarose gel electrophoresis of metagenomic DNA



Lane Information

1. lambda marker(48 kb)
2. Effluent soil DNA isolated through SDS-CTAB method
3. Effluent soil DNA after silica bead treatment
4. Effluent soil DNA isolated through MOBIO POWER MAX® soil DNA extraction kit
5. Fosmid control DNA
6. Silent valley soil DNA isolated through MOBIO POWER MAX soil DNA extraction kit
7. Silent valley soil DNA after silica bead treatment
8. Silent valley soil DNA isolated through SDS-CTAB method.

** Image inverted for clarity*

One of the drawbacks of SDS-CTAB method for soil DNA extraction is high humic content of the DNA preparation, which interferes with the library construction. Less than 0.8 µg/ml of humic acid is believed to inhibit the action of restriction enzymes even at high DNA concentrations (Tebbe and Vahjen, 1993). Even though most of the commercial kits give better quality humic free DNA, molecular weight of the DNA extracted is often ≤ 25kb, which makes it unsuitable for large insert library construction with Fosmid vectors which require a minimum of 35-40 kb size DNA. It was found that silica bead purification step after DNA extraction by SDS-CTAB method yields higher molecular weight DNA (~35kb) compared to the commercial DNA extraction kit. The

DNA was visibly free of humic acid and suitable for large insert library construction in Fosmid or BAC vectors.

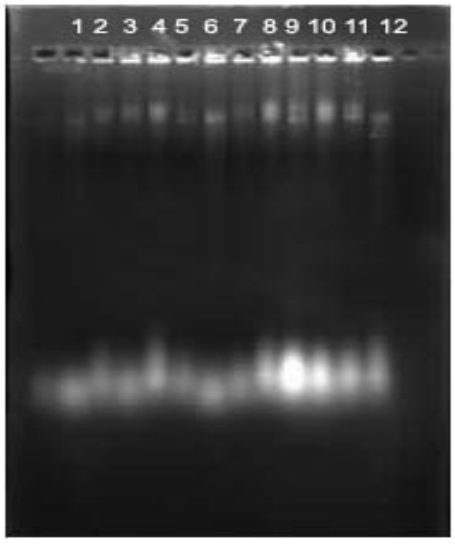
6.3.2. Construction of metagenomic library: Fosmid Library

The phage infected T1 resistant *Escherichia coli* cells plated on LB-chloramphenicol plates gave ~32000 clones of which 28000 were from Silent Valley Soil DNA and 4000 were from the effluent sludge DNA.

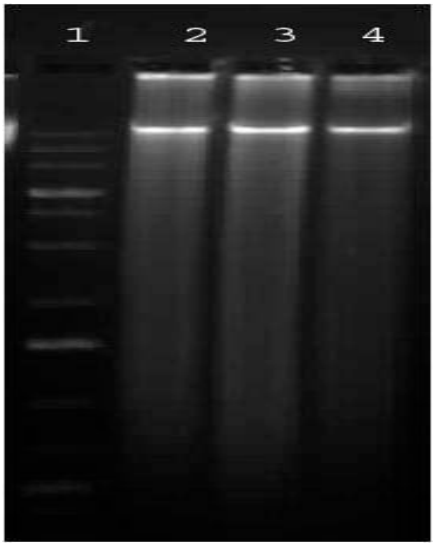
6.3.2.1. Confirmation of fosmid library

Fosmid library was confirmed by induction according to protocol from Epicentre. Overnight grown clones when treated with fosmid auto induction solution cause subsequent amplification of the fosmids to high copy number. Treatment with induction solution resulted in a higher copy number of fosmids as evidenced by agarose gel electrophoresis of fosmids isolated from the clones before and after induction (Figure 6.4A). Further confirmation of fosmid clones were done by fosmid isolation and insert size determination (Fig 6.4B)

Fig 6.4: Confirmation of Fosmid library by agarose gel electrophoresis of fosmid



Amplification of fosmids to high copy number on auto induction. Lanes- 1,3,5,7,9,11: fosmids from Uninduced random clones. Lanes – 2,4,6,8,10,12: fosmids from respective induced clones

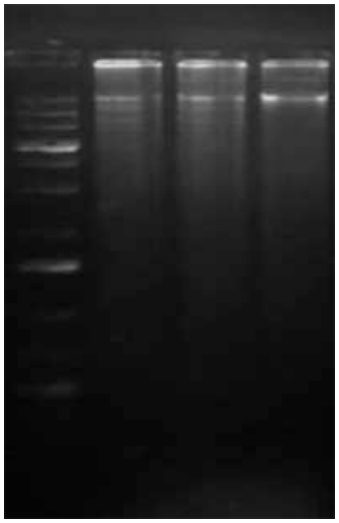


Confirmation of fosmid library by isolation of fosmids. Lanes – 1: 1kb plus DNA ladder, 2, 3, 4: fosmids isolated from random clones

6.3.2.2. Insert size determination of fosmid library

Agarose gel electrophoresis was conducted for *Hind* III digested fragments of Fosmid DNA and 5 -7 distinct bands were observed on the gel. Average insert size of the fosmids were analyzed from the size of the digested fragments with reference to the marker, and was found to be ~40kb. Gel picture showing the digested fragments is given below Fig 6:5.

Fig 6.5: Insert size determination of fosmids



Lane Information

1: 1kb plus Ladder (Fermentas)

2, 3, 4 – Hind III digested fosmids from random clones. 5-7 distinct bands are visualized in lane 2 and 3 and average insert size is calculated to be ~40kb

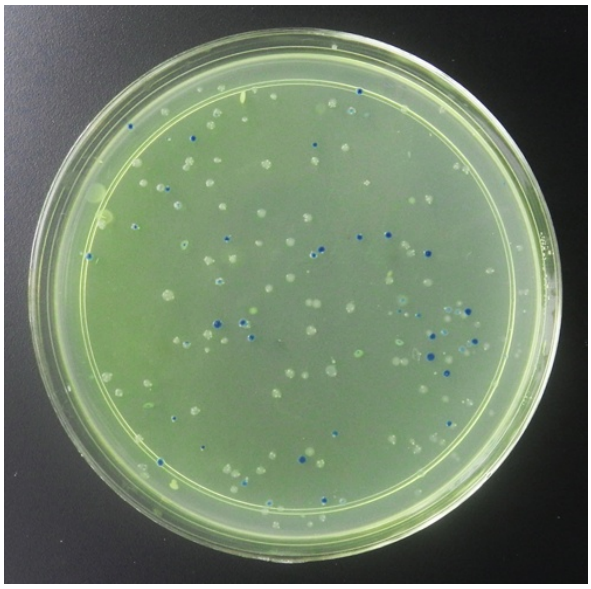
6.3.2.3. Screening of fosmid clones

32000 fosmid clones were screened for esterases of which 28000 clones were from Silent Valley metagenome and 4000 clones were from effluent sludge metagenome. No zone of clearance was observed with fosmid clones on LB-tributyrin agar plates from either Silent Valley metagenome or effluent sludge metagenome. Absence of positive result after screening huge number of clones indicated that the silent valley soil samples might not be the right choice of metagenome for screening lipases. The probability to get enriched with lipidic substrates to enhance the existence of lipase producers is comparatively less in forest soils. Even though the concept of metagenomics brings in the unprecedented chance to explore biomolecules, the selected source of metagenome is of at most importance.

6.3.3. Construction of metagenomic library: BAC Library

A total of 1369 BAC clones were obtained covering approximately 39Mb of the total metagenomic DNA. While 817 clones were obtained with the DNA extracted using commercial kit each containing ~25kb insert, 552 clones with ~35kb inserts were obtained using the DNA isolated by SDS-CTAB method. The constructed BAC library consisted of 967 clones from effluent sludge metagenome and 402 clones from Silent Valley metagenome.

Fig 6.6: BAC clones with soil DNA inserts (Representative Plate)

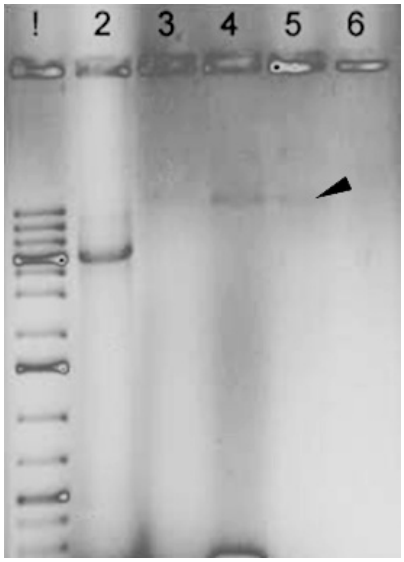


Blue white selection of clones with inserts inside BAC vector.
White clones have inserts in the BAC vector

6.3.3.1. Confirmation of BAC library

Plasmids isolated from randomly selected clones were run on agarose gel electrophoresis to look for shift due to inserts. Isolated plasmids showed shift in gel position compared to control BAC vector confirming the success of BAC library

Fig. 6.7. Confirmation of BAC library by isolation of BAC with insert from random transformed colonies



Lane Information

1: 1kb plus Ladder (Fermentas)

2: Undigested BAC vector (Control)

3, 4, 5: Plasmids isolated from randomly selected clones

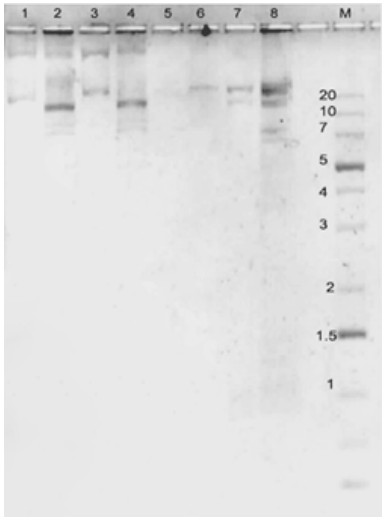
All plasmids isolated from clones had higher molecular weight than the control BAC indicating insertion of DNA. Shifted band indicated by arrowhead

** Image inverted for clarity*

6.3.3.2. Insert size determination of BAC library.

Plasmids from random clones on restriction digestion with *Hind* III gave distinct bands in agarose gel. The average insert sizes of the clones were calculated from the digested fragments as 30kb which was in the expected range (Fig 6.8).

Fig 6.8: Determination of the insert size in BAC library clones



Lane Information

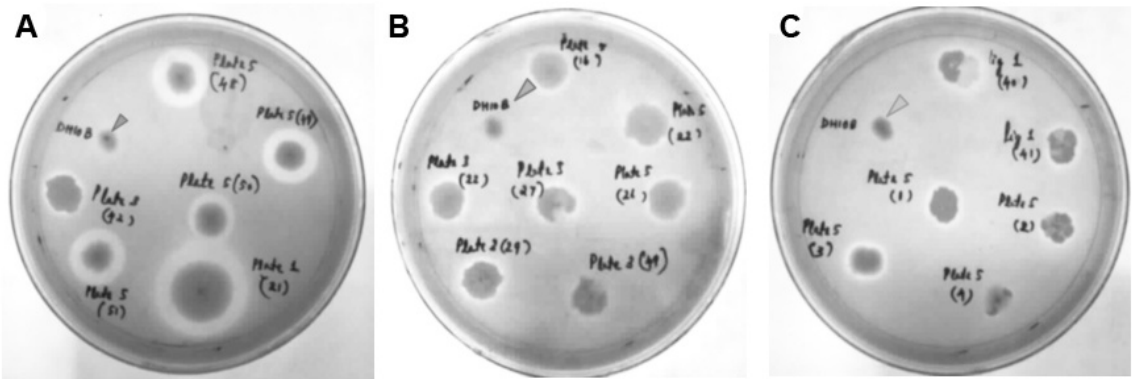
1: undigested plasmid from clone 1(40)
2: digested plasmid from clone 1(40)
3: undigested plasmid from clone 3(49)
4: digested plasmid from clone 3(49)
5: undigested plasmid from clone 5(50)
6: digested plasmid from clone 5(50)
7: undigested plasmid from clone 9(62)
8: digested plasmid from clone 9(62)
10: 1kb plus DNA ladder (Fermentas)

** Image inverted for clarity*

6.3.3.4.. Screening of BAC library

Nineteen clones from the effluent sludge metagenomic library and two clones from the Silent Valley (SV) soil metagenomic library showed clear zones in LB-tributyrin plates conferring esterase activity (Fig 6.9, Table 6.1), where as the host strain without insert (control) did not show any lipolytic activity.

Fig 6.9: Functional screening of BAC clones for lipase/esterase production in LB Tributyrin Agar



A, B, C: Testing of random clones for formation of clearance zones around colonies indicating esterase activity. Arrowheads mark the colony corresponding to the host strain (*E.coli* DH10B) without vector (control).

The total number of recombinant clones obtained for the BAC library was less (1369) which we presume to be due to the lower efficiency of electro-competent DH10B host cells and due to the low concentration of ligated inserts. Nevertheless, the number of lipase positive clones (19) were very high among the recombinant clones (1.96 %) indicating the high occurrence of lipolytic cultures in the effluent sludge. The occurrence of lipase positive clones as detected by the function based screening was 1 clone per 51 clones which is very high (Liaw *et al.*, 2010). Such high hit rates are associated with metagenomic libraries constructed from enriched sources (Mayumi *et al.,* 2008). The sample selected in this study can also be considered as an enriched source since the effluent sludge from soap factory presumably contains high amount of fat and oil waste. The high ratio of potent positive clones, irrespective of the smaller size of libraries make the soap factory effluent sludge an ideal source to screen for novel esterases through a metagenomic approach. On the other hand only two esterase positive clones were

obtained from the Silent Valley soil metagenomic clones. The lower percentage of positive clones from silent valley metagenome was expected based on the previous results of fosmid library screening. Even though silent valley soil metagenome might not be an appropriate source for esterases, the esterases obtained from this unexplored metagenome would be promising as far as novelty and diversity is concerned.

Table 6.1. Esterase positive clones obtained from BAC library

SL #	Clone ID	Source	Clearance Zone in LB-Tributyrin Agar
1	1(40)	Effluent Sludge	+
2	1(41)	Effluent Sludge	+
3	3(22)	Effluent Sludge	+
4	3(29)	Effluent Sludge	+
5	3(41)	Effluent Sludge	+
6	3(42)	Effluent Sludge	+
7	5(1)	Effluent Sludge	+
8	5(16)	Effluent Sludge	+
9	5(2)	Effluent Sludge	+
10	5(22)	Effluent Sludge	+
11	5(26)	Effluent Sludge	+
12	5(27)	Effluent Sludge	+
13	5(3)	Effluent Sludge	+
14	5(4)	Effluent Sludge	+
15	6(11)	SV Soil	+
16	6(12)	SV Soil	+
17	5(48)	Effluent Sludge	+
18	5(49)	Effluent Sludge	+
19	5(50)	Effluent Sludge	+
20	5(51)	Effluent Sludge	+
21	9(62)	Effluent Sludge	+

6.4. Conclusion

One Fosmid libraries and one BAC library each were constructed with DNA isolated from the Silent Valley Soils or the effluent sludge of a soap and detergent factory. Method of DNA extraction affected the quality of libraries and though the SDS-CTAB method has DNA of larger size the success of insertion was lesser. On the contrary DNA

prepared with commercial kits was of less molecular weight but provided better efficiency in library construction. Though about 32000 clones were obtained in Fosmid libraries, the number of clones obtained for effluent sludge DNA was lesser possibly due to some component which remained as impurities in the DNA. The number of clones obtained in BAC library was even lower, but out of the 1369 clones obtained in total, 967 were from the effluent sludge DNA. The number of lipase positive clones (19) were very high among the recombinant clones (1.96 %) indicating the high occurrence of lipolytic cultures in the effluent sludge. The occurrence of lipase positive clones as detected by the function based screening was 1 clone per 51clones which is very high. Such high hit rates are associated with metagenomic libraries constructed from enriched sources. The sample selected in this study can also be considered as an enriched source since the effluent sludge from soap factory presumably contains high amount of fat and oil waste. The results prove that soap and detergent factory effluent sludge can be a good source of lipolytic enzyme producing microorganisms.

Chapter 7

Studies on the properties of esterases from metagenomic clones

7.1. Introduction

Given the vast diversity of approximately 99% of the microorganisms remaining uncultivable, the probability of discovering novel compounds, which are potential drug candidates or biocatalysts are far more using a metagenomic approach. Metagenomics, along with high-throughput screening technologies, provides industry with an unprecedented chance to bring bio-molecules into industrial application (Patrick and Jürgen 2005). Esterases with novel properties are always in demand due to the immense number of synthetic reactions for which enzymatic routes are currently not available (Saxena *et al.,* 1999). Novel specificities, tolerance to extremes of pH, temperature and/or salt tolerance are features often desired in esterases/lipases. Since majority of the industrial applications of lipases are in synthesis of compounds and in esterifications, the stability of enzyme in organic solvents and its ability to act in presence of elevated concentrations of solvent is highly desired. Thermo stable lipases are important due to their applications in reactions which might needed elevated temperatures. Also some fats are in liquid form only when they are at higher temperatures and their hydrolysis can proceed only at liquid state (Gao *et al.,* 2000). Salt tolerant lipases are desirable in several industrial applications including food industry (Bruni *et al.,* 1982). Halo tolerant lipases and lipases with different fatty acid chain length specificities are desired for biotransformation reactions. Lipases stable under extreme conditions are also useful in waste water and oil spill treatments (Marhuenda-Egea and Bonete 2002; Pandey *et al.,* 1999).

In this study, the crude enzyme samples prepared from esterase positive clones obtained from the metagenomic library were studied for their biochemical properties like thermo, halo and solvent tolerance. Esterases isolated from some of the positive metagenomic clones possessed multiple important properties like thermo, halo and solvent tolerance.

7.2. Materials and methods

7.2.1. Enzyme production and assay

Enzyme production using the lipase positive clones was carried out using shake flask cultures. Hundred millilitres of LB medium containing 0.3% (v/v) tributyrin, in 500ml conical flasks was inoculated with 1ml of a 12h old culture of the prospective clone and was incubated at 37 °C for 72h at 200 rpm agitation. The culture broth was centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant was used as the crude enzyme preparation. Esterase activity was measured as the ability to hydrolyze para nitro phenyl butyrate (pNPB) by a modified method of Gupta et al. (Gupta *et al.*, 2006) as outlined in section 2.5.1. One unit of esterase activity was defined as the amount of enzyme liberating 1uM of pNP per milliliter per minute and was expressed as IU/ml.

7.2.2. Determination of the chain length preferences for the enzymes

The substrate chain length preferences for the enzyme preparations from different clones were evaluated using the p-nitrophenyl esters of fatty acids with carbon atom numbers ranging from 2 to 16 (p-nitrophenyl butyrate (C2), p-nitrophenyl valerate (C5), p-nitrophenyl octanoate (C8), p-nitrophenyl decanoate (C10), p-nitrophenyl laurate (C12), p-nitrophenyl myristate (C14) and p-nitrophenyl palmitate (C16)). A 0.5mM solution of the substrate was used for the assay as described in section 2.5.1.

7.2.3. Micro-diversity analysis on esterase positive clones.

16s rDNA were amplified using plasmids isolated from esterase positive clones as templates with the universal primers 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-GGTTACCTTGTTACGACTT-3' (Lane 1991; Turner *et al.*, 1999). Briefly, a 20µL PCR reaction mixture containing 5 ng of plasmid DNA, 0.1 µM universal bacterial primers, 2.5U Taq DNA polymerase, 25µM each deoxyribonucleotides, and 1X standard Taq buffer (all from Thermo Scientific, USA) were used. PCR conditions were as follows: Initial denaturation at 94 °C for 1 min, 25 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 1 min and a final extension at

72 °C for 5 min. The amplicons obtained were sequenced and BLAST analyses (Altschul *et al.,* 1998) were conducted to evaluate the bacterial diversity in the metagenome.

7.2.4. Biochemical properties of the enzyme

Crude enzyme extracts were used for testing different properties including temperature stability, solvent tolerance and halo tolerance as outlined below and similar to the methods specified under section 5.2.1

7.2.4.1. Temperature Stability

Temperature stability of the enzymes was studied by incubating the enzymes at different temperatures (50, 60, 70 and 80 °C) for 1h followed by activity assay using pNPB as substrate. Temperature stability was expressed as % of activity retained compared to that of the control which was incubated at room temperature (30 ± 2 °C) and assayed at 50 °C.

7.2.4.2. Solvent Tolerance

For estimating solvent tolerance, crude enzyme preparations were assayed in presence of 25% of the water miscible solvents methanol and acetone. The solvents were incorporated into the assay mixture so as to obtain the required final concentration and assays were performed at 50°C. Percentage activity retention was calculated against controls where solvents were not added in the assay mixture.

7.2.4.3. Halo Tolerance

Halo tolerance was studied by incorporating varying the concentrations of NaCl (0.5M - 2M) in the assay mixture and was expressed as % activity retention against controls lacking NaCl in the assay mixture.

7.3. Results and discussion

7.3.1. Substrate chain length specificities of esterases from metagenomic clones

Carboxyl esterases have been grouped into non specific esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) that differ primarily on their substrate specificities. While the latter cleaves esters of long chain fatty acids (>C14), the former hydrolyses water soluble short and medium chain (< C10) acyl esters (Uchida *et al.*, 2003; West *et al.*, 2009). Enzyme preparations from all the positive clones showed higher affinity for pNPB and the activity decreased sharply as the substrate chain length increased. None of the positive clones showed activity with esters of long chain fatty acids ≥ C16 (Table 7.1).

Table7.1 Esterase activity of enzymes from positive clones assayed with different pNP esters.

Clone ID	Enzyme activities obtained with different substrates (U/ml)						
	pNPB (C2)	pNPV (C5)	pNPO (C8)	pNPD (C10)	pNPL (C12)	pNPM (C14)	pNPP (C16)
1(40)	28	17	-	-	-	-	-
1(41)	22	10	-	-	-	-	-
3(22)	27	13	-	-	-	-	-
3(29)	35	24	-	-	-	-	-
3(41)	33	21	-	-	-	-	-
3(42)	30	19	-	-	-	-	-
5(1)	194	-	-	-	-	-	-
5(16)	72	-	-	-	-	-	-
5(2)	146	-	-	-	-	-	-
5(22)	94	-	-	-	-	-	-
5(26)	100	-	-	-	-	-	-
5(27)	109	-	-	-	-	-	-
5(3)	96	-	-	-	-	-	-
5(4)	102	-	-	-	-	-	-
6(11)	10	-	-	-	-	-	-
6(12)	8	-	-	-	-	-	-
5(48)	268	105	23	15	3.3	1.1	-
5(49)	80	62	19	23	21	7	-
5(50)	241	111	50	34	15	8	-
5(51)	259	50	17	15	17	-	-
9(62)	71	62	92	102	62	7	-

Chain length specificities of the positive clones indicated that most of the esterases captured from the sludge DNA were carboxyl esterases which showed higher affinity to p-nitrophenyl esters with lesser chain length (≤ C5). Five out of the 20 positive clones showed affinity to substrates with chain length ≥10C. Lipases prefer water-insoluble substrates, typically triacylglycerols with medium to long chain fatty acids (≥10 carbon atoms) (Jaeger and Eggert 2002; Pandey *et al.*, 1999; Uchida *et al.*, 2003). Esterases obtained in the study can therefore be considered as carboxyl esterases rather than lipases, since they preferentially hydrolyzed water soluble esters and triacylglycerols with fatty acids shorter than C5.

7.3.2. Micro-diversity analysis of esterase positive clones

Plasmids isolated from all the 21 esterase positive metagenomic (19 from effluent metagenome and 2 from silent valley metagenome) clones were amplified with 16S rRNA universal primers and 5 out of the 20 clones gave amplicons in the expected 1.5 kb size (Fig 7.1 and Table 7.2). On BLAST analysis 16s rRNA amplicon of clone 6 (11) showed 96% identity with *Paenibacillus* sp BIA1 and clone 6(12) showed 97% similarity with *Paenibacillus* sp KMS2U3. Both these clones were from the Silent Valley metagenome. Amplicons from clones 1(40), 5(1) and 5(4) on BLAST analyses showed significant alignments with uncultured bacteria. Amplicon from metagenomic clone 1(40), showed 96 % similarity with uncultured bacterium accession number HM812667.1 and that of 5(1) showed 95% similarity with the same uncultured bacterium clone described above. The clone 5(4) showed 95% similarity with uncultured bacterium clone with accession number JF241598.1. Preliminary analyses of the 16S rRNA sequence from the insert of the clones 1(40), 5(1) and 5(4) revealed homology with uncultured bacterium indicating the novelty of the obtained esterases, which is the prime gain in adopting a metagenomics approach.

Fig 7.1: 16S rDNA sequences amplified from lipase positive metagenomic clones

>Effluent_Clone_C1_40_16S_rDNA
GGGTCACTCATGTTAGTGGGATGGCAGCTCATCGCGATTACTAGCAATTCCGACTTCATGCAGGCGAGTTG
CAGCCTCAATCCGAACTGAGACCGGCTTTTTAGGATTCGTTCCACCTCGCGGTTTCACTGCCCGTTGTACC
GGCCATTGTAGTACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCC
GGTTTGTCACCGGCAGTCACCTTAAAGTGCCCACCCGAAGTGCTGGCAACTAAGATCAAGGGTTGCGCTCG
TTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCTCCTCTGTCCC
GAAGGAAAGGTACATCTCTGTACCGGTCAGAGGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGA
ATTAAACCACATACTCCACTGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTAC
TCCCCAGGCGGAGTGCTTAATGTGTTAACTTCGGCACCAAGGGTATCGAAACCCCTAACACCTAGCACTCA
TCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAG
TTACAGCCCCAGAGAGTCGCCTTCGCCACTGGTGTTCCCTCCACATATCTACGCATTTTCACCGCTACACG
TGGGAATTCCACTCTCCTCTTCTGCACTCAAGTCACCCAGTTTCCCAGTGCGATCCGGGGTTGAGCCCCGG
GATTAAACACCAGACTTAAATTGACCGCCTGCGCGCGCTTTACGCTCCATTATTTCCGGACTAACGCCTTG
CCCCCCTACTGTATTTACTGCTGGCTGCTGGCTACGTAATTAATCCTGGGGCTTTTCTTCTCAAGTACCCG
TCCCCTTGAGTAGCAGTTACTTCTCCCATCCGTTTCTTTCCCTGGGCAATGAACTTTTAGATTCTGAAAAC
CTT

>Effluent_Clone_5_1_16S_rDNA
CTATCGACTTGAGTGAGATGGCAGCTGATCGCGATTACTAGCAATTCCGACTTCATGCAGGCGAGTTGCAG
CCTGCAATCCGAACTGAGACCGGCTTTTTAGGATTCGTTCCACCTCGCGGTTTCACTGCCCGTTGTACCGG
CCATTGTAGTACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGG
TTTGTCACCGGCAGTCACCTTAAAGTGCCCACCCGAAGTGCTGGCAACTAAGATCAAGGGTTGCGCTCGTT
GCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCTCCTCTGTCCCGA
AGGAAAGGTACATCTCTGTACCGGTCAGAGGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAAT
TAAACCACATACTCCACTGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTC
CCCAGGCGGAGTGCTTAATGTGTTAACTTCGGCACCAAGGGTATCGAAACCCCTAACACCTAGCACTCATC
GTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTA
CAGCCCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATATCTACGCATTTCACCGCTACACGTGGAAT
TCCACTCTCCTCTTCTGCACTCAAGTCACCCAGTTTCCAGTGCGATCCGGGGTTGAGCCCCGGGATTAACC
ACCAGACTTAAATGACCGCCTGCGCGCGCTTTACGCCCAATAATTCAGACAACGCTTGCCCCCTACGAAAT
AACGCGGCTGCTGGCTCGTAGTTAGCCGGGGCTTTCTTCTCAAGTATACGTCACCTTGAGAGCTGTTACTC
CTCCCAAGCGTTCTTCCCTGGGCAACAGACGTTTACGAATCCGAAAATCCTTCCTTCACTCATGCGGACAT
TGCCTCCGGTCAGCACTTTCGCCCTATTGCGGAAAAATCCCTACAGATTACT

>Effluent_Clone_5.4_16S_rDNA
TTGGAAAAATGCATCTGTTTTGACTAGCGGCGGACGGGTGAGTAATACTTAGGAATCTGCCTATTAATGGG
GGACAACATCTCGAAAGGGATGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCACTTGTGACC
TTGCGTTAATAGATGAGCCTAAGTCGGATTACCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACCATCTG
TAGCGGGTCTGAGAGGATGAACCGCCACACTGGGACTGACACACGGCCCATACTCCTACGGGAGGCAGCAG
TGGGGAATATTGGACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTATGGTTG
TAAAGCACTTTAAGCGAGGAGGAGGCTCTTCTAGTTAATACCTAGGATGAGTGGACGTTACTCGCAGAATA
AGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGC
GTAAAGCGTGCGTAGGCGGCTTTTTAAGTCGGATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGA
TACTGGGAAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTATAGATCTGG
AGGAATACCGATGGCGAACGCAGCCATCTGGCCTAACACTGACGCTGAGGTACGAAAGCATGGGGGAGCAA
ACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCTTTGAGGCTTTT
TTGACGCACCTAACGCCATACTTATACCGCCTGGGGAGCACGGTCGCGAGACTAAAACTCAATGAATTGAC
GGGGGCCCGCACAGGCAGGCGGAAGCATGTGGTTTAATTCGAATGCAACGCGAAGAACCCTTACTGGACCT
TGTACATAGGTACGAACTTTCCCAAAGATGGATTTGTGGACTTGGGGATCTTTACATTCAGAGTGCTGCAA
TGGCTT

>SilentValley_Clone_6_11_16S_rDNA
GGTGAGTAACACGTAGGCAACCTGCCCTCAAGCTTGGGACAACTACCGGAAACGGTAGCTAATACCGAATA
GTTGTTTTCTTCTCCTGAAGAAAACTGGAAAGACGGAGCAATCTGTCACTTGGGGATGGGCCTGCGGCGCA
TTAGCTAGTTGGTGGGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGC
CTGACGGAGCAATGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGCCAGGGAAGAACGCT
TGGGAGAGTAACTGCTCTCAAGGTGACGGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCATTTAAGT
CTGGTGTTTAATCCCGGGGCTCAACCCCGGATCGCACTGGAAACTGGGTGACTTGAGTGCAGAAGAGGAGA
GTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTG
GGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGAGTGCTAGGTGTTAGGGGTTTCGATACCCTTGGTGCCGAAGTTAACACATTAAGCACTCCGC
CTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAGTATGTG
GTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCTCTGACCGGTACAGAGATGTACCT
TTCCTTCGGGACAGAGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCACTTCGGGTGGGCACTCTAAGGTGACTGCCGGTG
ACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTACTAC
AATGGCCGGTACAACGGGCAGTGAAACCGCGAGGTGGAACGAATCCTAAAAAGCCGGTCTCAGTTCGGATT
GCAGGCTGCAACTCGCCTGCATGAAGTCGGAATGCTAGT

>SilentValley_Clone_6_12_16S_rDNA
GGTGAGTAACACGTAGGCAACCTGCCCTCAAGCTTGGGACAACTACCGGAAACGGTAGCTAATACCGAATA
GTTGTTTTCTTCTCCTGAAGGAAACTGGAAAGACGGAGCAATCTGTCACTTGGGGATGGGCCTGCGGCGCA
TTAGCTAGTTGGTGGGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGC
CTGACGGAGCAATGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGCCAGGGAAGAACGCT
TGGGAGAGTAACTGCTCTCAAGGTGACGGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCATTTAAGT
CTGGTGTTTAATCCCGGGGCTCAACCCCGGATCGCACTGGAAACTGGGTGACTTGAGTGCAGAAGAGGAGA
GTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTG
GGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGAGTGCTAGGTGTTAGGGGTTTCGATACCCTTGGTGCCGAAGTTAACACATTAAGCACTCCGC
CTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAGTATGTG
GTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCTCTGACCGGTACAGAGATGTACCT
TTCCTTCGGGACAGAGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCACTTCGGGTGGGCACTCTAAGGTGACTGCCGGTG
ACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTACTAC
AATGGCCGGTACAACGGGCAGTGAAACCGCGAGGTGGAACGAATCCTAAAAAGCCGGTCTCAGTTCGGATT
GCAGGCTGCAACTCGCCTGCATGAAGTCGGAATGCT

Table 7.2: Micro-diversity analysis of esterase positive clones for metagenomic libraries

Clone ID	Source	Amplification*	Homology with reported cultures	% Similarity
1(40)	Effluent Sludge	Yes	Uncultured bacterium HM812667.1	96 %
1(41)	Effluent Sludge	-		
3(22)	Effluent Sludge	-		
3(29)	Effluent Sludge	-		
3(41)	Effluent Sludge	-		
3(42)	Effluent Sludge	-		
5(1)	Effluent Sludge	Yes	Uncultured bacterium HM812667.1	95%
5(2)	Effluent Sludge	-		
5(3)	Effluent Sludge	-		
5(4)	Effluent Sludge	Yes	Uncultured bacterium JF241598.1.	95%
5(16)	Effluent Sludge	-		
5(22)	Effluent Sludge	-		
5(26)	Effluent Sludge	-		
5(27)	Effluent Sludge	-		
5(48)	Effluent Sludge	-		
5(49)	Effluent Sludge	-		
5(50)	Effluent Sludge	-		
5(51)	Effluent Sludge	-		
6(11)	Silent Valley	Yes	*Paenibacillus* sps BIA1	96%
6(12)	Silent Valley	Yes	*Paenibacillus* sp KMS2U3	97%
9(62)	Effluent Sludge	-		

7.3.3. Studies on the properties of the enzyme

7.3.3.1. Temperature stability

Enzyme extracts from all the 21 positive clones were tested for temperature stability by assaying the enzyme after 1h incubation at elevated temperature. Enzymes from 10 clones among the 21 positive ones, ie the clones - 1(40), 1(41), 3(22), 3(29), 3(41), 3(42), 5(49), 6(11), 6(12) and 9(62) showed detectable pNPB hydrolysis activity after incubation at elevated temperatures. Enzymes from the clones 3(41), 6(11), 6(12) and 9(62) showed higher activity after incubation at 80 °C, compared to the control which

was incubated at room temperature (30 ± 2 °C). These clones thus retained 100 % activity and those from clones 3(22) and 3(42) retained < 90% activity and clones 1(40) and 3(29) retained < 80% activity after high temperature treatment (80°C for 1h), indicating the thermo stability of these enzymes. Enzymes that could cleave medium chain fatty acid esters, except that from clone 9(62), were found to be temperature sensitive and not stable at temperatures above 50 °C. Among the 10 clones which showed activity after incubation at elevated temperatures, four were found to be thermo-stable and did not loss any activity after the high temperature treatment (Table 7.3).

Table 7.3: Temperature stability of esterases from selected metagenomic clones

Clone ID	Enzyme Activity (IU/ml) and Percentage activity Retention (% AR) of esterases at different temperature								
	Control (30 °C)	50 °C	% AR	60 °C	% AR	70 °C	% AR	80 °C	% AR
1(40)	41	36	88	36	88	35	85	36	88
1(41)	52	45	87	44	85	33	63	42	81
3(22)	39	36	92	37	95	36	92	36	92
3(29)	70	54	77	53	76	55	79	52	74
3(41)	37	46	124	43	116	45	122	42	116
3(42)	41	34	83	35	85	35	85	39	95
5(49)	126	107	85	83	66	63	50	71	56
6(11)	8	8	100	9	113	8	100	9	113
6(12)	6	6	100	7	117	7	117	8	133
9(62)	51	98	192	87	171	83	163	76	149

7.3.3.2. Solvent tolerance

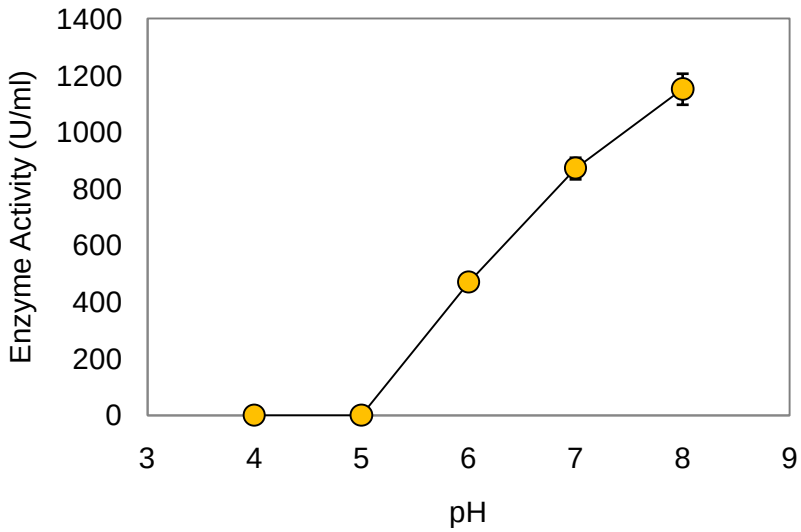
Out of the esterases tested for solvent sensitivity, those from clones 1(40), 1(41), 3(22), 3(29), 3(41), 3(42), 5(49), 5(50), 5(51), 6(11), 6(12) and 9(62) showed varying levels of tolerance in 25 % acetone while all these enzymes except the one from clone 5(50) showed tolerance in 25% methanol. Use of carboxyl esterases in non aqueous media in presence of organic solvents, especially for the resolution of racemic mixtures by transesterification, enantio and regio selective hydrolysis is well known (Bornscheuer, 2002; Gupta and Roy 2004; Illanes *et al.*, 2008; Torres *et al.*, 2011). Esterases obtained from selected clones showed activity in presence of methanol and acetone. One of the

clones 6(11) retained 70% and 40% esterase activity whereas, an esterase from clone 9(62) could retain 69% and 39% activity in presence of 25% methanol or acetone respectively (Table 7.4). Thermo tolerant esterases with solvent tolerance and pH tolerance are always in demand as it can be used in chemical synthesis, especially in the production of racemates and also in biotransformation and bioremediation applications (Adamczak M., 2003; Panda and Gowrishankar 2005; Kongpol *et al.,* 2008). Enzymes from all the clones were active at pH 8.0. The activity of the esterase from 9(62) which showed the highest activity in presence of solvents and at elevated temperature was tested at different pH till pH 8.0 Activity could not be studied beyond this pH as the substrate pNPB was readily hydrolyzed at higher pH even without enzyme action. This enzyme showed progressively higher activities with increase in pH upto pH 8.0 (Fig 7.2). Increased activity at high pH (8.00) for enzymes from all the positive clones indicated the peculiarity of the DNA source for the metagenomic library. Except for clones 6 (11) and 6 (12), the DNA source was effluent sludge from a soap and detergent factory, which was alkaline.

Table 7.4: Solvent tolerance of esterases from positive clones

Clone ID	Enzyme Activity (IU/ml) and % Activity Retention in presence of 25 % of solvent				
	Control	Methanol	% AR	Acetone	% AR
1(40)	55	13	25	13	25
1(41)	34	28	82	15	44
3(22)	40	18	44	14	35
3(29)	54	22	41	22	41
3(41)	51	20	39	21	41
3(42)	46	17	37	22	48
5(49)	93	54	58	41	44
5(50)	191	0	0	41	21
5(51)	100	42	42	42	42
6(11)	10	7	70	4	40
6(12)	8	5	63	4	50
9(62)	78	54	69	28	36

Fig 7.2: pH dependence of esterase from metagenomic clone 9(62)



7.3.3.3. Halo tolerance

Esterases that can act in high salt concentrations are always in demand as they can be used for bioremediation of effluent and in waste water treatment (Marhuenda-Egea and Bonete 2002). Eight out of the 20 positive clones were found to be halo tolerant. All the eight clones i.e. -1(40), 1(41), 3(22), 3(29), 3(41), 3(42), 5(49) and 9(62) showed elevated esterase activity in presence of NaCl, compared to the control (Table 7.5).

Table 7.5: Halo tolerance of esterases from metagenomic clones

Clone ID	Control*	0.5M	% AR	1.0 M	% AR	1.5M	% AR	2.0M	% AR
1(40)	35	50	143	52	149	52	149	50	143
1(41)	29	84	290	37	128	39	134	40	138
3(22)	38	ND	ND	55	145	56	147	52	137
3(29)	48	63	131	72	150	69	144	65	135
3(41)	46	57	124	56	122	53	115	55	120
3(42)	37	40	108	54	146	51	138	50	135
5(49)	85	107	126	110	129	ND	ND	128	151
9(62)	79	104	132	104	132	ND	ND	110	139

* Without NaCl, ND – not determined

Morimoto *et al* 2006, had explained the conformational changes and re-activation of esterase at higher salt concentration which could result in an increase in esterase activity

in the presence of high NaCl concentrations (Morimoto *et al.*, 2006). Structural changes in the enzyme are proposed in presence of high salt concentrations due to enhanced hydrophobic interactions and reduced electrostatic repulsions and this may lead to enhanced activity in presence of salt (Szeltner and Polgár 1996). Interestingly, the enzymes that showed higher salt tolerance were the same ones which were temperature tolerant. Esterase activities of clones 5(49) and 9(62) were higher than those of the controls even at 2M NaCl, which indicated their possible halophilic nature and potential applications in industry. Esterase from clone 9(62) appeared highly promising with its high temperature stability, solvent tolerance and enhanced activity in presence of salt. An esterase with all the three properties would be highly valuable in industrial applications.

7.4. Conclusion

The occurrence of esterase producing clones in the metagenomic library from the effluent sludge DNA was very high, indicating an exceptionally high occurrence of lipolytic organisms in the soap factory effluent stream. Enzymes from many of the positive clones had important properties like high thermo stability and tolerance towards solvents and high salt concentrations, which are particularly useful for industrial applications. Enzyme from clone - 9(62) even had an enhanced activity at 80 °C and at 2M NaCl concentration indicating its potential for further exploration, especially for industrial applications. Even though esterases from the Silent Valley metagenome clones - 6(11) and 6(12) had high thermo tolerance and solvent tolerance, the esterase activity was significantly less compared to enzymes from other clones. Majority of the esterases obtained from the metagenomic libraries can be included under carboxyl esterases (EC 3.1.1.1) rather than lipases (EC 3.1.1.3) based on the substrate specificity and chain length preferences shown. None of the positive clones showed activity with esters of long chain fatty acids indicating that the positive clones harbored non-specific esterases and not lipases. Diversity analyses of the 16S rRNA sequence from the insert of the clones 1(40), 5(1) and 5(4) revealed homology with uncultured bacterium indicating the novelty of the obtained esterases, which was the primary purpose in adopting a metagenomics approach.

Chapter 8

Purification and characterization of esterase from metagenomic library clone and cloning of the partial gene sequences

8.1. Introduction

Protein purification is essential for the study of its function, biochemistry and the structure. Purification of an esterase from a metagenomic library clone is no different than purification the enzyme from a host system where it is cloned. Functionally active enzyme from a metagenomic library implies that the enzyme is expressed by the host organism and a functional protein is being formed. Common strategies for protein purification like concentration and chromatography are employed in purification of proteins from metagenomic clones. Purification strategies involving concentration (by ammonium sulfate/acetone precipitation or ultra filtration) followed by affinity, ion exchange or hydrophobic interaction chromatography are commonly employed in the case of clones from metagenomic libraries (Mohamed *et al.,* 2013; Shao *et al.,* 2013; Peng *et al.,* 2014).

While about 21 clones positive for esterase activity were obtained altogether from 4 metagenomic libraries including two fosmid and two BAC libraries each from two different source DNA, an initial screening of the properties of the enzymes elaborated by them indicated that some of these clones harbor potent esterase genes whose products are unique with respect to their thermo, solvent and halo tolerance. The clone 9(62) produced an esterase which was thermo stable even up to 80 °C, retained activity in 25 % of polar water miscible solvent and had a high halo tolerance (Chapter 07). This enzyme was purified to homogeneity and its properties were studied. PCR amplification of the esterase gene was performed and the partial gene sequence was cloned and sequenced to understand further about the enzyme.

PCR methodologies based on specific (Xu *et al.,* 2010) as well as degenerate primers (Neugnot *et al.,* 2002; Bigey *et al.,* 2003; Zhang *et al.,* 2007) were used in majority of the successful cloning strategies for esterases. Degenerate primers are typically designed based on the consensus motiff (G-X-S-X-G) present in the active site, which is conserved in most esterase enzymes. PCR methodology had also been used to

prospect for novel esterase genes directly from environment DNA despite the low homology observed between esterases (Bell *et al.,* 2002). This was made possible by extensive analysis of conserved regions and careful primer designing based on oxyanion hole and active site of lipases. Amplification of esterase gene using primers designed corresponding to the peptide sequence after N terminal sequencing is also popular (DeAngelis *et al.,* 2007). In this study, partial amplification of esterase gene from potent clone 9(62) was done using degenerate primers designed based on oxyanion hole and active site of esterases. A similar strategy was also employed for amplification and cloning of the partial gene sequence of the enzyme from clone 6(11) from Silent Valley metagenome.

8.2. Materials and Methods

8.2.1. Production of esterase by submerged fermentation

Enzyme production using the esterase positive clone 9(62) was carried out using shake flask cultures. Hundred milliliters of LB medium containing 0.3% (v/v) tributyrin, in 500 ml conical flasks was inoculated with 1ml of a 12h old culture of 9(62) and was incubated at 37 °C for 72h at 200 rpm agitation. The culture broth was centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant was used as the crude enzyme preparation. Esterase activity was measured as the ability to hydrolyze para nitro phenyl butyrate (pNPB) as outlined under section 2.5.1. One unit of esterase activity was defined as the amount of enzyme liberating 1uM of pNP per milliliter per minute and was expressed as IU/ml.

8.2.2. Purification of esterase from clone 9(62)

Crude esterase produced as above (1000ml) was concentrated to 300ml through ultrafiltration using a 50 kD cut off membrane and was then subjected to ammonium sulphate precipitation. The active fraction was dialysed against 50 mM Tris to remove the salt content. Enzyme was further purified by ion exchange chromatography using Q Sepharose® (GE Lifesciences, USA). Elution of bound proteins was done with a step gradient of 0.1 -1.0M NaCl in 50 mM Tris buffer (pH 8.0) at a flow rate of 1ml min^{-1}.

Fractions of 2ml were collected and assayed for esterase activity. The active fractions were pooled, dialyzed against 0.02M Tris buffer to remove salts. The homogeneity of the purified protein was analyzed by Native PAGE. After electrophoresis for the analysis of esterase pattern, activity staining (Zymogram) was performed with 0.1M methyl umbelliferyl butyrate as described under section 2.6.1. This was followed by silver staining, with a modified protocol of Blum et al (1987) as outlined under section 2.6.1. Approximate molecular weight of the esterase was estimated based on electrophoretic mobility in SDS-PAGE (Laemmli, 1970).

8.2.3. Studies on the properties of the purified esterase

8.2.3.1. Thermostability

The thermostability of purified esterase from clone 9(62) was studied by incubating the enzyme at different temperatures (40, 50, 60 and 70 °C) for 1h followed by assay of the enzyme at 50 °C. The activities obtained were compared to the control enzyme incubated at room temp (30 ± 2 ^{o}C) and assayed at 50 ^{o}C.

8.2.3.2. Solvent Tolerance

Purified esterase was analyzed for tolerance to methanol by incorporating the solvent into the assay mixture so as to get the required final concentration of 25%. Enzyme assays were performed at its optimum temperature of 50 ^{o}C.

8.2.3.3. Halo Tolerance

Since the crude enzyme preparation showed halotolerance, purified 9(62) esterase was also tested for tolerance to salt by performing the esterase assays in presence of varying concentrations of sodium chloride. NaCl was added to the assay mixture to obtain final concentration in the range 0.05-2.0 M and the assays were performed at 50 °C.

8.2.4. Partial amplification of esterase gene in clone 9(62) using PCR

Partial amplification of the esterase genes harbored by clones - 9(62) and 6(11) was performed using degenerate primers designed based on the conserved sequences of the oxyanion hole and active site of esterases so as to amplify a small region covering these conserved sites in esterases (Bell *et al.,* 2002). Three forward primers were designed complementary to the nucleotide sequences corresponding to the conserved oxyanion hole signature and four reverse primers were designed complementary to the conserved active site signature of esterases (Table 8.1). Combinations of these primer sets were used for PCR amplification of partial gene sequences of esterases. Sequencing of the amplicons was outsourced to a service provider (Scigenom, Kochi, India).

Table 8.1: Primers used for amplification of esterase genes

Primer name	Direction	Complimentarity	Sequence (5' -3')
OXF1	Forward	Oxyanion hole signature	CCYGTKGTSYTNGTNCAYGG
OXF2	Forward	Oxyanion hole signature	CCRATMRTWYTNGTNCAYGG
OXF3	Forward	Oxyanion hole signature	CCKYTWGTKYTNATHCAYGG
ACR1	Reverse	Active site signature	AGGCCNCCCAKNGARTGNSC
ACR2	Reverse	Active site signature	AGRCCNCCCAKRCTRTGNSC
ACR3	Reverse	Active site signature	AGGCCRCCNTGNGARTGNSC
ACR4	Reverse	Active site signature	AGGCCNCCNTGRCTRTGN

8.3. Results and Discussion

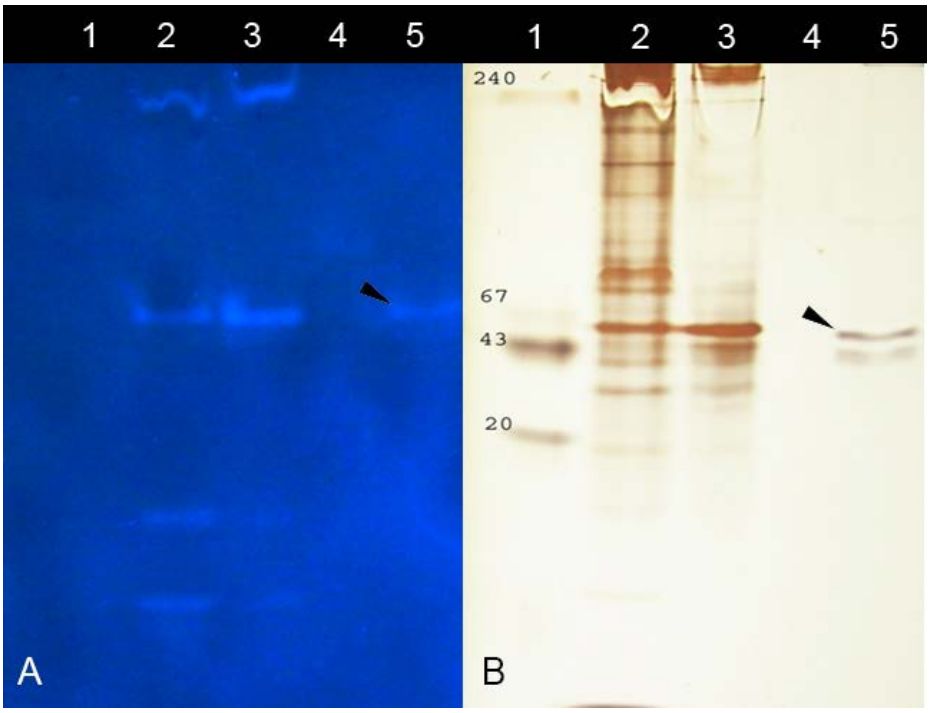
8.3.1. Purification of esterase from clone 9(62)

Fractional precipitation of crude esterase concentrated through ultra filtration (300 ml) resulted in the maximal esterase activity being recovered in the 50% ammonium sulphate fraction. The dialyzed fraction was further purified through anion exchange chromatography using Q Sepharose ®. The summary of the purification is presented in Table 8.2. Native PAGE followed by Zymogram analysis (Fig 8.1A) and silver staining (Fig 8.1B) confirmed the purity of the protein. It may also be noted that the crude enzyme as well as the ammonium sulfate fraction contained more than one esterase indicated by the presence of activity bands other than the one detected for ion exchange fraction in the Zymogram (Fig 8.1A). It may be speculated that the insert in clone 9(62) harbored more than one gene for esterase.

Table 8.2 Summary of purification of esterase from clone 9(62)

	Volume (ml)	Esterase activity (U/ml)	Total activity (U)	Protein content (mg/ml)	Total protein (mg)	Specific activity	Fold purification
Crude enzyme	1000	39	39000	0.15	150	260	100
50% Ammonium sulfate fraction	10	98	980	5.7	57	17	7
Ion exchange fraction	8	5	40	0.16	1.3	31	12

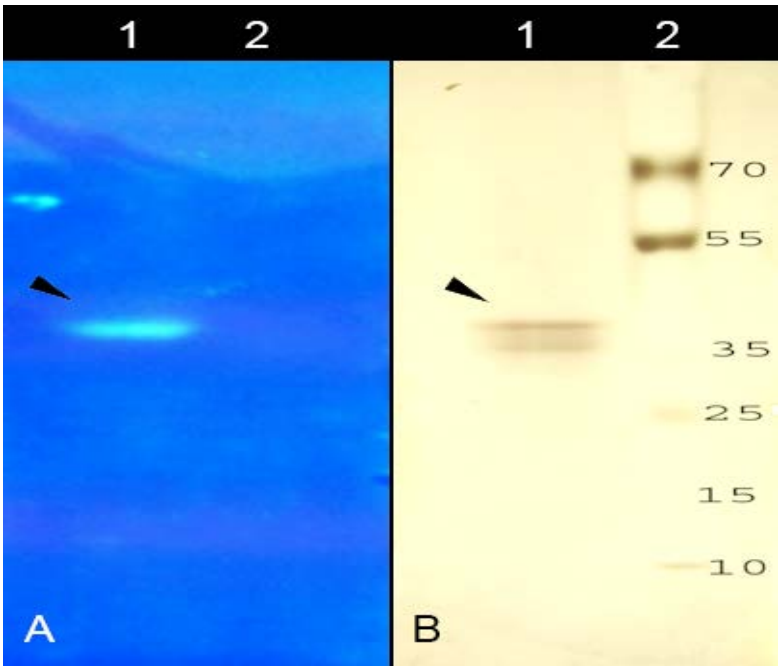
Fig 8.1: Native PAGE and Zymogram analysis of esterase from clone 9(62)



Zymogram (A) and Silver stained (B) Native PAGE Gels. Lanes - 1: Native PAGE marker, 2:Crude enzyme, 3:50% Ammonium sulfate fraction , 5: Ion exchange fraction
*Arrow heads indicate the purified 9(62) esterase

Approximate molecular weight of the esterase was determined through non reducing SDS-PAGE to be around 40kDa (Fig 8.2A&B). Both the zymogram and silver stain images showed that the protein is purified to homogeneity and probably is monomeric since only one band at ~ 40KDa was detected in either case. This 40kDa esterase was designated as *EST*1.

Fig 8.2: SDS-PAGE and Zymogram analysis of purified esterase from clone 9(62)

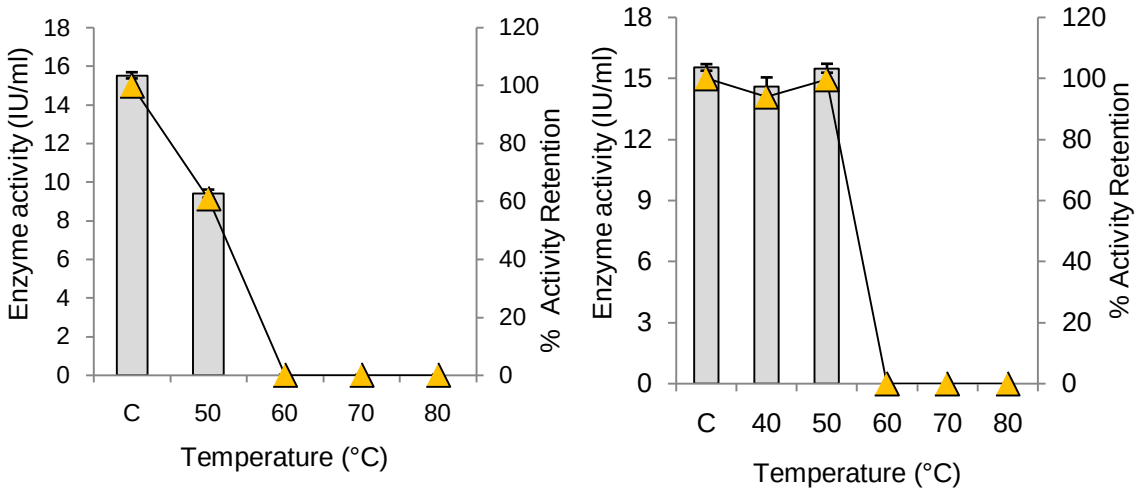


Zymogram (A) and Silver Stained (B) SDS PAGE Gel. Lanes -1: Purified esterase (EST1) from 9(62) 2: SDS PAGE marker
*Arrow heads indicate the purified 9(62) esterase

8.3.2. Studies on the properties of the purified esterase.

A significant change in esterase properties was observed after purification. Purified esterase was found to be active only up to 50°C and a significant stability loss was observed. Purified esterase showed only 61% activity after treating in 50°C for 1 h (Fig 8.3A & B). Previous studies with crude esterase preparation from clone 9(62) showed an optimum enzyme activity at 50 °C and 80% activity retention after treating the enzyme at 80 °C for 1h which may qualify it as a thermo stable enzyme. The radical activity loss observed may be due to the conformational change occurred during stringent purification conditions, or due to the fact that the other esterase proteins detected in the crude enzyme did not co-purify with the ~40kDa *EST*1 esterase. These esterases would have contributed to the enhanced temperature tolerance and stability.

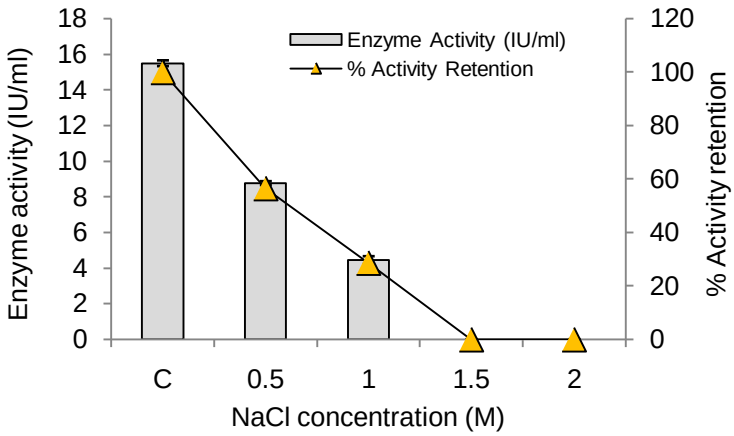
Fig 8.3: Temperature Tolerance and Temperature Stability of the 40kDa *EST1*



Temperature tolerance (A) and Temperature stability (B) of EST1. Bars indicate the Enzyme activity and lines the % activity retention in comparison to the control-C

Similarly, though the crude esterase preparation was found to be stable in 2.0 M NaCl concentration, the purified *EST*1 was observed to have reduced tolerance to salt. Prolonged exposure of the enzyme to high salt concentration during ammonium sulphate precipitation as well as ion exchange chromatography is reported to impair the enzyme activity and stability (Dako *et al.*, 2012). Almost half of the esterase activity was lost in 0.5 M NaCl and a steady decrease in enzyme activity was observed with increasing salt concentration (Fig 8.4). In contrast to crude enzyme preparation, no esterase activity was observed at 1.5 M NaCl concentration. While crude esterase preparation retained 70% activity in 25% methanol, only 61% activity retention was observed with purified esterase in the same reaction conditions.

Fig 8.4: Salt tolerance of the purified 40kDa *EST1*

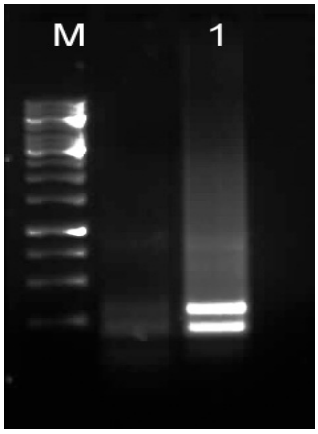


8.3.3. Amplification of the partial gene sequences of esterase from potent clones

8.3.3.1. Amplification of partial gene sequence of esterase from clone 9(62) and sequencing

Esterase genes in the BAC inserts were amplified with degenerate primers based on sequences corresponding to the oxyanion hole and active site signatures in different combinations. Amplicons of ~250bp size was obtained with plasmids from the esterase positive clone 9(62) (Fig 8.5)

Fig 8.5: Confirmation of esterase gene in the BAC vector of clone 9(62) as insert



Lane Information

M: 1kb ladder (Fermentas)

1: PCR amplification of esterase gene from BAC vector insert of 9(62)

The primer combinations that returned amplicons from the vector were *OXF1-ACR4* (Table 8.1). The ~250bp amplicon from the insert of clone 9(62) was sequenced and the partial gene sequence is shown below.

```
>Esterase_Oxf1-Acr4_Clone_9(62)
TCACATATCGCATACTGGCTGCATGGATTCAGGAAAGCCCAGCAGGTAACATGTCAGCTTGAAAG
TACTCAGACTTGACCCGTCAACTACGCCAAAACTGGAGGTCTGGGAACAGCAAATGCGTGAACAG
ATTGATGGGATTGACGAAAATAGTATTTTTATTGCCCACAGCCACGGCGGCCTAAAAATTCGGGA
TATTTGCTGGCGCATTATAGAAGGGTCTTGATGCGCAGGCCGTCCGGGGCCCGTACACCCCTGAA
TTTCAAGTGGGCGCGACATGTACATTTTTTTCACACGACGGTGTTATTTTTTTT
```

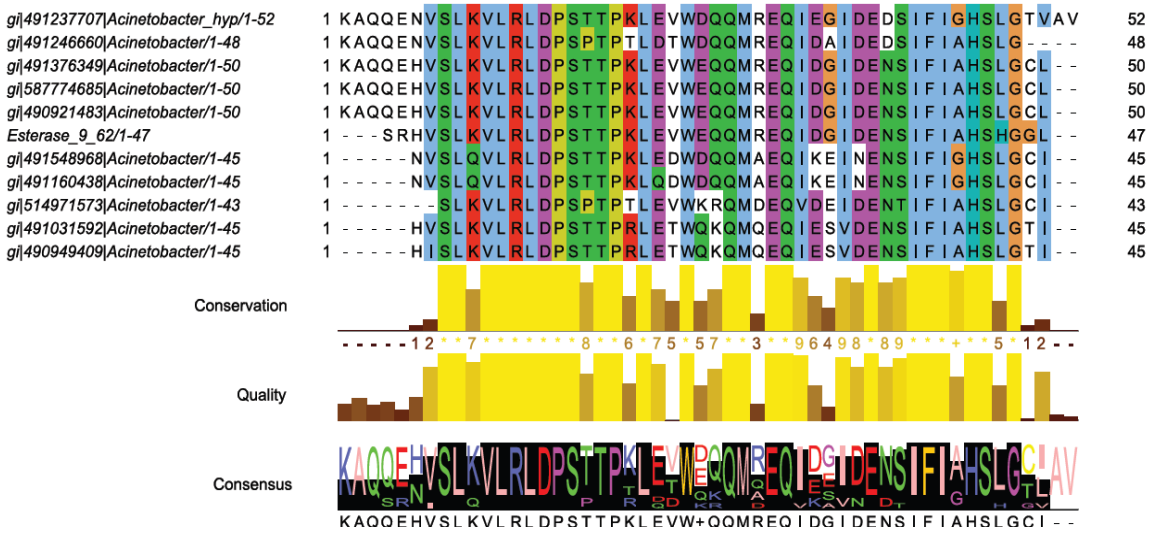
On BLAST analysis with “blastx”, the translated protein sequence corresponding to the amplicon showed significant alignments with hypothetical proteins predicted as carboxyl esterases and esterases confirming that the insert indeed carries carboxyl esterase gene(s) (Fig 8.6)

Fig 8.6: Significant alignments produced by BLAST (blastx) analysis

A: translated protein sequence

```
HISHTGCMDSGKPSRHVSLKVLRLDPSTTPKLEVWEQQMREQIDGIDENSIFIAHSHGGLKIRDI
CWRIIEGS-CAGRPGPVHP-ISSGRDMYIFFTRRCYFF
```

B. Alignment of the translated protein sequence with the hits having significant homology

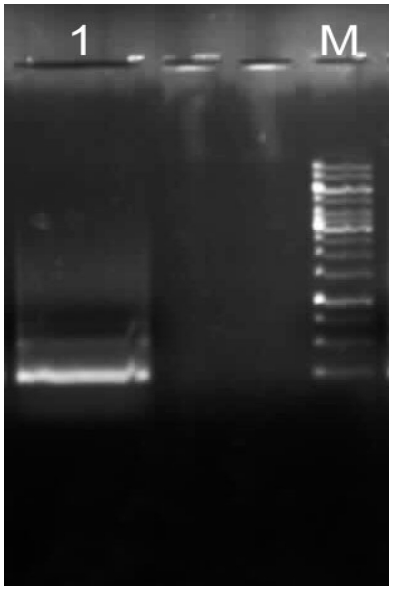


The entire top hits for the translated protein sequence was to hypothetical proteins from various *Acinetobacter* strains which was annoted as possible carboxyl esterases. The sequence and the major hits were scanned against InterPro database of protein family signatures (Jones *et al.,* 2014) to deduce the sequence matches against known family/domain signatures. The sequences to which the translated protein sequence matched had the protein of unknown function DUF1234 hydrolase (IPR010662) family signature match. DUF 1234 is a family of proteins with unknown hydrolase activity but with a α,β hydrolase fold suggesting an enzymatic function. Also almost all the hits returned by BLAST was either a hypothetical protein annotated as carboxyl esterase or an esterase confirming that the insert indeed carries an esterase gene. Also the fact that the hits are not directly towards well known esterases indicate that the esterase may be novel.

8.3.3.2. Amplification of partial gene sequence of esterase from clone 6(11) and sequencing

Amplicons of ~250bp size was obtained with plasmids from the esterase positive clone 6(11) obtained from silent valley effluent sludge (Fig 8.7).

Fig 8.7: Confirmation of esterase gene in the BAC vector of clone 6(11) as insert



Lane Information

M: 1kb ladder (Fermentas)

1: PCR amplification of esterase gene from BAC vector insert of 6(11)

The primer combinations that returned amplicons from vector were *OXF1-ACR4*. The 250bp amplicon from the insert of clone 6(11) was sequenced and the partial gene sequence is shown below.

>Esterase_Oxf1-Acr4_Clone_6(11)
CTGGATTTCTTGGCGCATGGCTCACTGGAAGGACAGCACCACAATCCGGATATTACAACAGGGAT
CGCGGCTTATCACTATGGCATGTCTTATTTACAGCATAAGCTTTCGCCAGAAGTAATCGGTCGGC
CGTTTTTCATTAACTTGTCCATCGCGCCATTATTTCCATATGCTTTTGCCCACAGCCACGGCGGC

On BLAST analysis, the sequence showed 70% similarity to GDSL hydrolase which is a diverse family of lipases or esterases of *Paenibacillus* sp. Distantly related members of the α-β hydrolase superfamily share similar conserved motifs with the lipases/esterases in the oxyanion hole and active site, thus making these genes targets for gene prospecting by PCR (Bell *et al.*, 2002).

8.4. Conclusion

Twenty one esterase positive clones were obtained from four metagenomic libraries (2 fosmid and 2 BAC libraries) totaling about 33500 clones out of which 19 were from effluent sludge metagenome and 2 from silent valley soil DNA. Esterases from all the clones were screened for properties like tolerance to temperature, solvent and salt and many of them were found to possess important features like tolerance to temperature, solvents and/or salt. Crude esterase from clone 9(62) showed temperature, salt and solvent tolerance was purified to homogeneity. The molecular weight of the protein was determined to be ~ 40kDa. Partial amplification of the esterase from clone 9(62) and 6(11) were attempted using degenerate primers designed based on homologous sequences in carboxyl esterases which included the oxyanion hole and active site. Amplicons of ~250bp obtained were sequenced. BLAST analysis of translated gene sequence from clone 9(62) showed significant sequence similarity with *Acinetobacter* sp. hypothetical proteins annotated as carboxyl esterases and esterases from *Campylobacter* sp. The partial gene sequence of 6(11) showed 70% sequence similarity with GDSL hydrolase which is a diverse family of lipases or esterases of *Paenibacillus* sp. The presence of homologous sequences in carboxyl esterases justified the use of these degenerate primers for partial amplification of the esterase gene.

Chapter 9

Studies on magnetic nanoparticle immobilized esterase for biotransformation reactions

9.1. Introduction

Immobilized Lipases/esterases are considered as suitable biocatalysts for several synthetic and biotransformation reactions. The use of immobilized enzymes facilitates the effective recovery, reuse and long term stability of the enzymes. The chemo-, stereo-, and regio-, specific reactions catalysed by lipases make them inevitable candidates for industrial biotransformations (Gross *et al.*, 2001). However, the high cost and increased reaction times limits the usage of free enzymes in organic synthesis. Enzyme immobilization can overcome these limitations and provide stable and cost effective catalyses (Bornscheuer, 2003; Pavlidis *et al*., 2013; Lee *et al.*, 2013). Immobilization of enzymes on magnetic nanoparticles (MNPs) is now widely practiced and has several advantages over conventional immobilization strategies (Wang *et al.*, 2012; Yu *et al.*, 2013; Mahmood *et al.*, 2013). Immobilization on MNPs allow higher binding due to more specific surface area, lower mass transfer resistance and lesser fouling, besides the major advantage of selective separation of catalyst from reaction mixtures, long term storage, and re-use (Dyal, 2003; Xie and Ma, 2009). Therefore, the immobilization of lipases on MNPs broadens their applications in biotransformation reactions.

Esters of short chain fatty acids and alcohols are important components of natural flavors and aromas (Mahmood *et al.*, 2013). Immobilized lipase mediated synthesis of esters in solvent free systems are important because of avoidance of solvent toxicity and easy enzyme recovery (Guvenc *et al.*, 2002). Biodiesel production is another major biotransformation mediated by lipases. Fatty Acid Methyl Esters (FAMEs) which form the biodiesel can be synthesized from vegetable oils by acid/alkali catalyzed transesterification reactions with methanol/ethanol. Enzymatic transesterification for production of FAMEs have several advantages including purer products, easy seperation of glycerol byproduct, higher compatibility with feed stocks containing higher levels of free fatty acids, and ability to operate at ambient conditions (Yu *et al.*, 2013). While enzymatic processes offer these advantages, the major limitation is the cost of enzymes; and immobilization can address this issue very effectively (Ferrario *et al.*, 2013). Though lipases immobilized on MNPs for biodiesel production have been reported by many

researchers (Xie and Ma, 2009; Karimi *et al.,* 2013; Yu *et al.,* 2013), novel specificities and enzyme properties are always desirable in biocatalysts since the lipase catalyzed reactions are in several cases chemo-, stereo- or regio- specific.

In the present study, glutaraldehyde functionalized magnetite nanoparticles were used for immobilizing the isolated novel *Pseudozyma sp* esterase NII 08165. (Deepthy *et al.,* 2014). The efficiency of MNP immobilized *Pseudozyma* sp esterase in synthesis of the flavor compound ethyl acetate and in the transesterification of sunflower oil for biodiesel production was studied and was compared with an immobilized commercial lipase preparation.

9.2. Materials and Methods.

9.2.1. Preparation of magnetite nanoparticles

$FeCl_3 \cdot 6H_2O$, $FeCl_2 \cdot 4H_2O$, $(NH_4)_2OH$ solution (25% w/w), 37 % HCl, 25 % Glutaraldehyde, Absolute alcohol and solvents for biotransformation reactions were obtained from Merck (Germany). (3-Aminopropyl)-triethoxysilane APTES (99%), was purchased from Sigma-Aldrich (USA). Commercial Lipase (Steapsin) was purchased from SRL (India). All aqueous solutions were prepared using deionized water. The stock solutions of 2M $FeCl_2.4H_2O$ and 1M $FeCl_3.6H_2O$ prepared in 2M HCl was mixed in the ratio 1:4 respectively and 0.7M aqueous ammonia solution was added drop wise into the solution with constant stirring in nitrogen atmosphere. A black precipitate of magnetite was formed which was washed thoroughly with deionized water and was separated by magnetic decantation (Fig 9.1) The magnetic nanoparticles (MnPs) were allowed to dry at 50 °C and were stored until used.

Fig 9.1: Preparation of Magnetic Nanoparticles (MNPs)

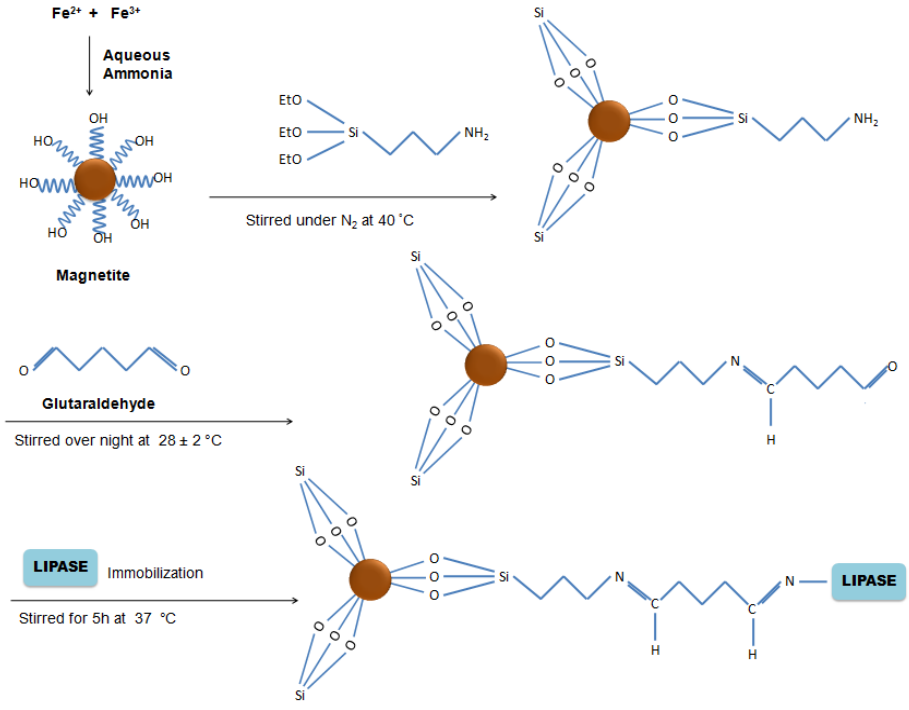
Magnetite nanoparticles in solution | MNPs in presence of Magnet | Dried MNPs

9.2.2. Surface functionalization and immobilization of esterases on MNPs

Dried pellets of magnetite nanoparticles (0.845g) were suspended in a 1:1 mixture of ethanol and water (1:1) and the mixture was sonicated for 30 min with 10 second on and off pulse to get a uniform suspension. APTES (3.405ml) was added to this mixture such that the molar concentration of magnetite to APTES was 1:4 (Shen *et al.*, 2004). The reaction was performed under nitrogen atmosphere at 40 °C for 2h with constant stirring. APTES modified magnetite particles (153 mg) were dispersed in 6 ml of 5% aqueous glutaraldehyde solution and was reacted overnight at room temperature (30 ± 2 °C) with constant stirring. The magnetite particles after reaction was washed 3× with deionized water and was used for immobilization of esterases.

Pseudozyma sp.NII 08165 esterase production, partial purification and assays were performed as outlined in the chapters 2 and 3. Enzyme immobilization was performed by incubating 15, 25 or 50 mg of MNPs with 200U of partially purified *Pseudozyma* sp. esterase in a total volume of 1ml Tris buffer (pH 8.0, 50mM). A schematic diagram showing the steps involved in magnetic nanoparticle preparation is shown in Figure 9.2.

Figure 9.2: Schematic representation showing preparation of lipase immobilized MNPs



Enzyme loaded MNPs were then separated using a magnetic field and were washed repeatedly with Tris buffer (pH 8.0, 50mM) to remove unbound enzyme. Binding efficiency was calculated as percentage of original enzyme activity used for immobilization. A control reaction was performed similar to above with commercial lipase (Steapsin, SRL India). Total protein in the reaction mixture before and after immobilization was assayed by Bradford's method (Bradford, 1976).

9.2.3. Characterization of the Magnetic Nanoparticles

Enzyme immobilized magnetic nano particles were passed through 0.22 μ filters to remove particle aggregates formed due to the mutual attraction of magnetic particles and the filtrate was analysed by Dynamic Light Scattering and Atomic Force Microscopy. DLS analyses were carried out with a Zetasizer Nano S (Malvern Instruments, UK) at 25 °C. Average of at least ten measurements was taken. The samples were prepared in cyclohexane (1×10^{-5} M) and kept for 6h at room temperature before measurements. For the light induced morphology evaluation, samples were irradiated using the required wavelength at different time intervals, and the measurements were performed after the samples were thoroughly mixed and equilibrated for 2min. The average hydrodynamic radii were calculated from Stork-Einstein equation

$$(Rh = kBT/(6\pi\eta D)).$$

Atomic Force Microscopy images were recorded under ambient conditions using a NTEGRA (NT-MDT) operating with a use tapping mode regime. Micro-fabricated TiN cantilever tips (NSG10) with a resonance frequency of 299 kHz and a spring constant of 20-80 Nm^{-1} were used. AFM section analysis was done offline. Infrared spectra were recorded using Fourier transform infrared spectrometer (Shimadzu Prestige 21). The sample and KBr were pressed into a tablet and the magnetization curves of samples were measured with a vibrating sample magnetometry at room temperature. Infra red spectrum of uncoated MNPs, MNPs with ligand attachment, enzyme immobilized MNPs were analyzed.

9.2.4. Studies on the applications of lipase immobilized MNPs for biotransformations.

9.2.4.1. Synthesis of flavor compound-Ethyl Acetate

Lipase immobilized ferrofluid was repeatedly washed with hexanol to remove water, and a 25ml reaction was set up in 1:3 ratio of glacial acetic acid and ethanol in hexanol. A mixture containing 4.4 mol dm^{-3} (6.41 ml) of ethanol and 1.46 mol dm^{-3} (2.09 ml) of glacial acetic acid in hexanol (17 ml) were incubated at 40 °C with 500U of *Pseudozyma* esterase immobilized on MNPS. Aliquots were taken every 24 hrs for analysis. Ester concentrations in the sample were analyzed by gas chromatography (Chemito 8600, India) with a flame ionization detector, and using a Porapak Q ® column. Nitrogen flow rate through the column was 2.0 ml min^{-1}. Injection port and detector temperature were set at 100 °C and 250 °C respectively.

9.2.4.2. Biodiesel production

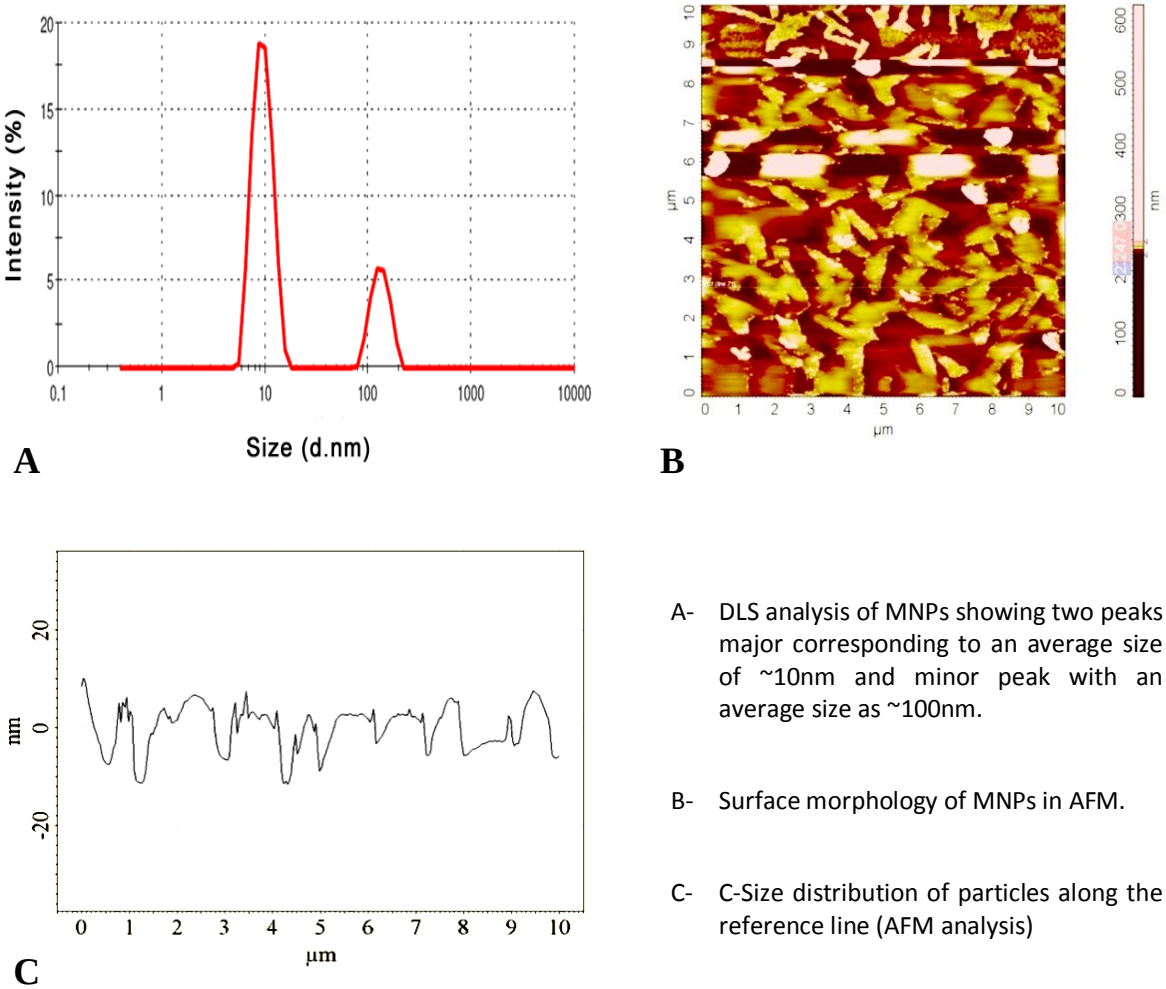
The reaction mixture for transesterification consisted of sunflower oil and dried methanol in the ratio 1:3. Methanol was maintained in the reaction mixture at 25 % v/v level along with hexanol as it was found that the *Pseudozyma* lipase could retain its enzyme activity at that concentration of methanol (Chapter 5, Deepthy *et al.,* 2014). Five hundred milligram of Molecular Sieve 4A (SRL, India) were added to the reaction mixture to control the excess water formation during transesterification reaction as it may shift the equilibrium to hydrolysis over synthesis. The mixture was incubated at 50 °C for 72h with reciprocal shaking. A control reaction with commercial lipase was also performed similarly. Hundred micro liter samples were taken each 12h for GC analysis. Fatty acid methyl ester (FAME) analysis was performed using gas chromatograph (GC2010, Shimadzu Japan) equipped with FID, using a DB225 capillary column (Agilent technologies USA). The column temperature was programmed to rise at the rate of 5°C/min from 160°C (2 min) to 230 °C and was then held at 230 °C for 20min. The carrier gas flow rate was 1 ml/min and the sample injection volume was 1µl. FAME composition was analyzed comparing against standard FAMEs and were expressed as relative area percentage. The stability and reusability of lipase immobilized MNPs stored at 4 °C were analyzed by repeating the reaction after 30 days.

9.3. Results and Discussions

9.3.1. Production and properties of magnetic nanoparticles

Magnetic nanoparticles (magnetite) were successfully synthesized by co-precipitating $FeCl_2$ and $FeCl_3$ in presence of ammonia. The nanoparticles were washed with deionized water and dried at 50 °C and stored in an air tight container. The magnetite nanoparticles appeared as a dark suspension which could move under an external magnetic field (Fig 9.1). Dynamic Light Scattering analyses indicated two peaks of size intensity corresponding to 10 nm and 100 nm (Fig 9.3A). Atomic force microscopy confirmed that the particle size ranges from 10-20nm (Fig 9.3B & C).

Figure 9.3: Characterization of the magnetic nanoparticles



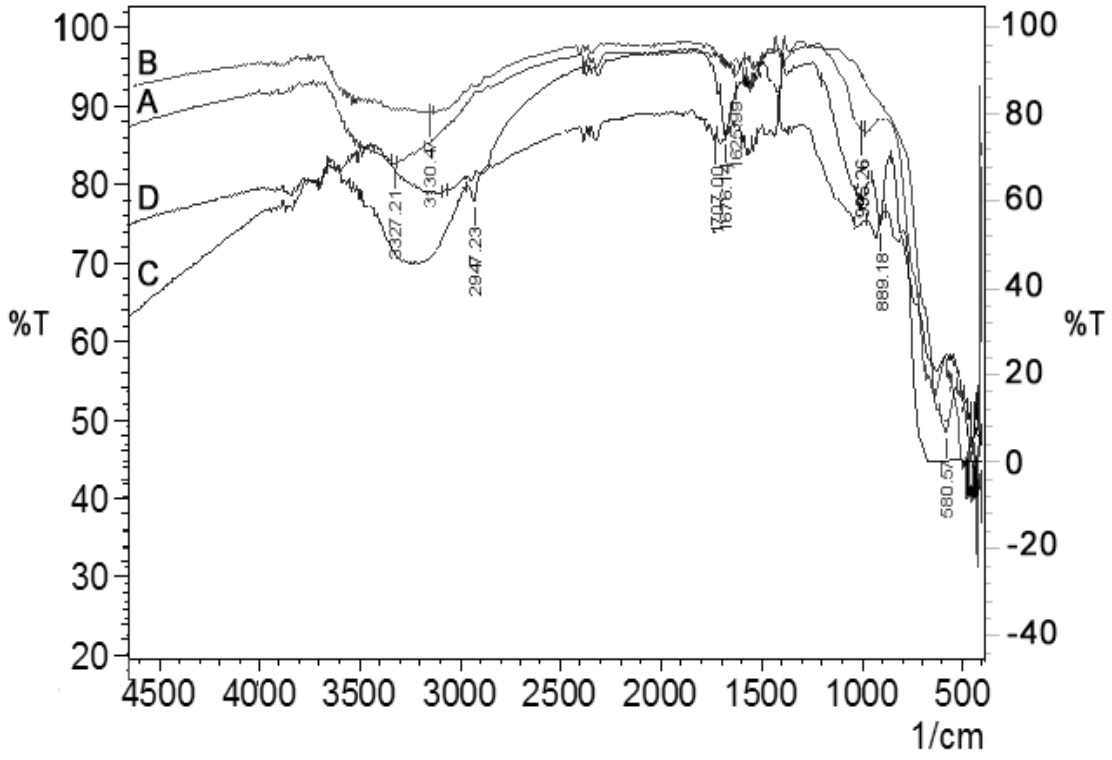
A- DLS analysis of MNPs showing two peaks major corresponding to an average size of ~10nm and minor peak with an average size as ~100nm.

B- Surface morphology of MNPs in AFM.

C- C-Size distribution of particles along the reference line (AFM analysis)

FTIR spectra of uncoated, amino functionalized, glutaraldehyde attached and lipase immobilized MNPs were compared to determine the efficiency of surface functionalization and immobilization (Fig 9.4). FTIR band at short wave numbers near 580cm^{-1} due to the vibrations of Fe-O bonds is characteristic of magnetite and was common for all the spectra. The introduction of APTES to the surface of MNPs was confirmed by the band at 995cm^{-1} attributed to Si-O stretching. The broadened peak around 3200cm^{-1} was probably due to free amino groups which is overlapped by the O-H stretching vibration. In glutaraldehyde attached MNPs an additional peak was observed at 1676 cm^{-1} due to >C=N bond formation. The band at 2947cm^{-1} and near 900cm^{-1} was due to the C-H stretching of aldehyde group and Si-CH_2 bonds respectively. Additional imino group formation during lipase immobilization caused a shift in band position to 1700 cm^{-1} confirming the covalent binding of the enzyme to MNPs.

Figure 9.4: FTIR analysis of free, surface modified and lipase immobilized MNPs.



A- Free magnetic nanoparticles. **B**- APTES modified MNPs. **C**- APTES modified and glutaraldehyde functionalized MNPs. **D**- MNPS with esterase immobilized through covalent bonding with glutaraldehyde.

9.3.2. Efficiency of Esterase immobilization on magnetic nanoparticles

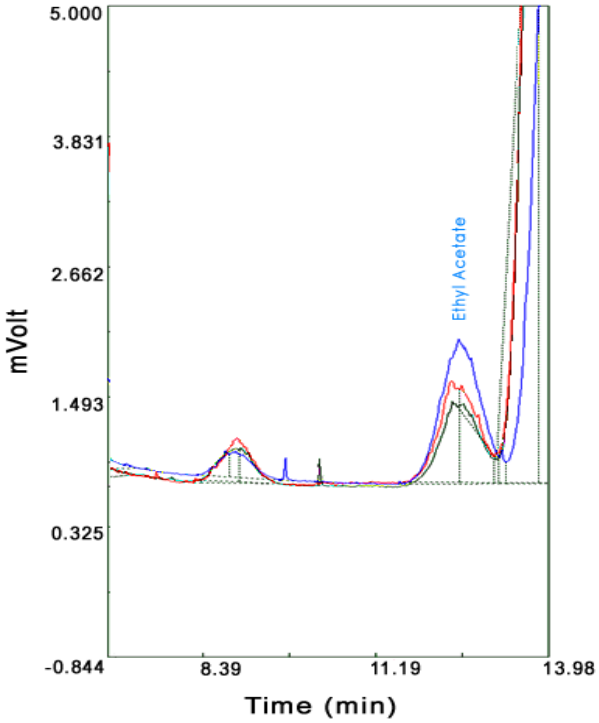
The binding efficiency of esterase to the MNPs was evaluated by incubating 200 U of enzyme with different concentrations of 15, 25 or 50mg of MNPs. This corresponded to enzyme concentrations of 13, 8 and 4 U/mg of MNPs respectively. The enzyme activities retained for 15, 25 and 50 mg MNPs were 91, 103 and 106 U for the *Pseudozyma* esterase whereas, these were 131, 163 and 190 U respectively or the commercial lipase. Apparently, a higher initial loading of enzyme had resulted in a higher amount of bound enzyme per unit amount of MNPs, but the binding efficiency was proportionate to the MNP concentration. With higher quantity of MNPs, more enzymes were bound and the binding efficiencies were 46, 52 and 53% respectively when 15, 25 or 50 mg MNPs were used for immobilization of *Pseudozyma* esterase. These values were 66, 82 and 95 % when the commercial lipase was used. A direct correlation between enzyme activity and amount of MNPs cannot be expected as only those enzymes which are bound and still active can give positive results with the enzyme assay. During immobilization, the enzyme covalently binds the glutaraldehyde moiety with free amino terminal ends (Gupta *et al.,* 2013). The binding is non-specific with respect to the amino acid position and enzyme activity loss due to conformational change can occur if the imino bond is formed with any of the amino acids in or around the active site.

9.3.3. Studies on application of MNP immobilized esterases

9.3.3.1. Synthesis of Ethyl Acetate

Pseudozyma sp. esterase immobilized on MNPs was tested for the synthesis of flavour compound – ethyl acetate, and for trans-esterification of sunflower oil to produce biodiesel. MNP immobilized *Pseudozyma* esterase could successfully catalyze the synthesis of ethyl acetate and a steady increase in the ethyl acetate concentration was observed with increase in the duration of incubation. The amount of ethyl acetate produced after 24, 48 and 72 h of incubation were 6.24, 8.17 and 12.82 mM respectively (Fig 9:4)

Figure 9.5: Ethyl Acetate Synthesis by MNP immobilized *Pseudozyma* sp. esterase



Analysis of Ethyl acetate formation by MNP immobilized esterase

Black Line – 24h incubation

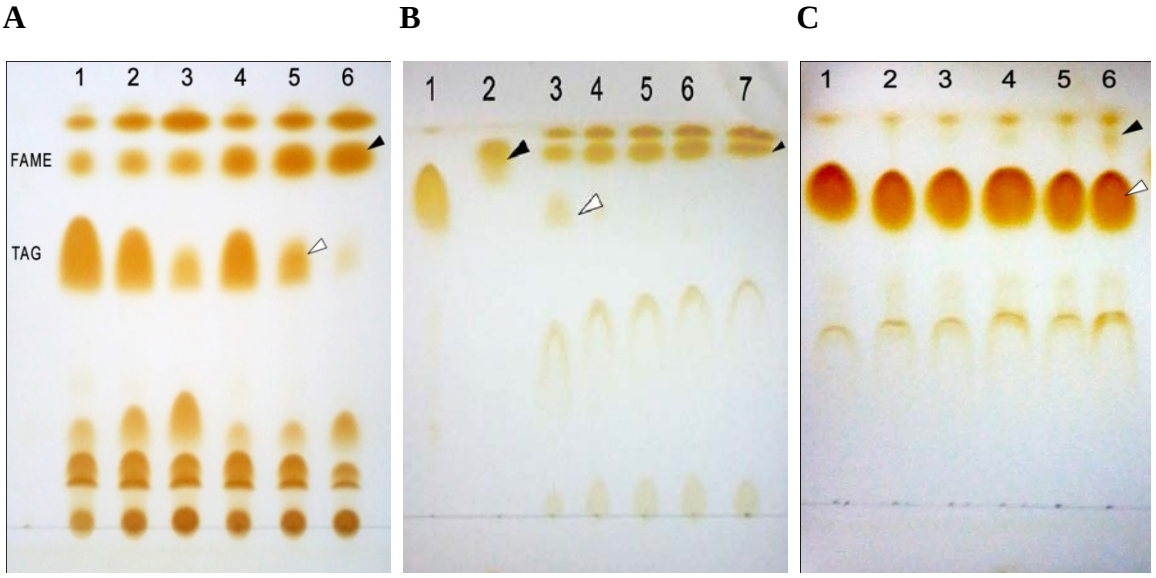
Red line – 48h incubation

Blue line – 72h incubation

9.3.3.2. Biodiesel production using MNP immobilized Pseudozyma sp. esterase

TLC analysis of the FAMEs generated by MNP immobilized esterase indicated that the enzyme concentration and reaction time significantly influenced FAME production. Generally the time required to complete enzymatic transesterification ranges from 8-20h depending on the efficiency of the enzyme (Akoh *et al.*, 2007). While 200 U of commercial lipase immobilized on MNPs (50mg) could effectively convert a significant amount of sunflower oil to methyl esters in 60h (Fig 9.6A), a high enzyme concentration (~2500 U) was required in the case of *Pseudozyma* esterase for achieving a positive result (Fig 9:6B). Increase in enzyme loading resulted in a significant reduction in reaction time in the case of commercial enzyme which was reduced to 24h from 60h (Fig 9:6C).

Fig 9.6: Transesterification of sunflower oil by MNP immobilized esterases for biodiesel production



Transesterification of sunflower oil using 4U/mg (**A**) or 50U/mg steapsin (**B**), and 50 U/mg *Pseudozyma* esterase (**C**) immobilized MNPs. Black arrow heads indicate Fatty acid Methyl Esters (FAME) and white arrowheads show the unreacted Triacyl glycerol (TAG). Lane Information: (**A**) 1, 2, 3 reactions using 100 U Steapsin for 12, 24 and 48h respectively, 4, 5, 6 – reactions using 200U of Steapsin 12, 24 and 48h respectively. (**B**) 1- Unreacted oil (negative control), 2- Standard FAME (Positive control), 3 -7: *Steapsin* (2500 U) catalyzed reactions for 12, 24, 36, 48 and 72h respectively. (**C**) 1-6: *Pseudozyma* esterase (2500 U) catalyzed reactions for 12, 24 , 36, 48, 60 and 72h respectively.

The FAME profiles of enzymatically synthesized biodiesel as well as the one synthesized by chemical catalysis are provided in Table 9:1. The major fatty acids in sunflower oil linoleic (~60%), oleic (~30%), palmitic (~7%) and stearic acids (~5%) has stereo specificity in distribution on the glycerol moiety. The polyunsaturated fatty acids (linoleic and linolenic) predominantly occupy *sn2* position, while saturated fatty acids (palmitic and stearic) are concentrated in the primary positions *sn1* and *sn3*, and monoenoic acids (oleic) are relatively evenly distributed (Zimmerman and Fick, 1973; Garces and Martinez-Force, 2005). The expected high percentage of linoleic acid was not observed in the GC profile of both *Pseudozyma* esterase and Steapsin mediated catalysis. This could be due to the position specificity of the enzyme. The regio-specificity of Steapsin for fatty acids at *sn-1/3* positions and a few acyl migrations of fatty acids from *sn2* to *sn1/3* positions were attributed as the reasons for the high concentration of palmitic acid and low concentration of linoleic acid in the GC profiles of FAMEs from sunflower oil in another report (Karupaiah and Sundram, 2007).

Table 9.1: FAME profile of the biodiesel generated by transesterification using MNP immobilized esterases.

FAME Type	Percentage of different FAME types synthesized with different catalysts		
	Pseudozyma esterase MNP*	Steapsin MNP*	Alkali catalyst
C 16:0	52.3	46	15
C 18:0	5.4	12	23
C 18:1	6.8	7	9
C 18:2	-	15	39
C 18:3	-	21	13

* ~2500 U of esterase immobilized on 500mg MNPs

The specific reaction catalyzed by an esterase/lipase under given conditions is its finger print and esterases with different and distinct fingerprints are required for catalyses which demand different specificities (Chandler *et al.*, 2001). Results indicate that the position specificity of *Pseudozyma* sp esterase may be expected at the primary positions (*sn1/3*) similar to steapsin though it had a distinctly different reaction fingerprint. In chemical catalysis where position specificity of fatty acids is of no significance, the concentration of linoleic acid outweighed other fatty acids (Table 9:1). The immobilized enzymes retained 100% activity even after storage for 3 months at 4 °C. This ensures the stability and scope in reusing immobilized magnetic nanoparticles for biotransformation reactions.

9.4. Conclusion

Applicability of MNP immobilized *Pseudozyma* sp esterase for biotransformation reactions were demonstrated. Despite the lower yield of products with this enzyme, the immobilized enzyme retained 100% activity even after prolonged storage at 4 °C. The enzyme had a distinctly different reaction fingerprint which might be useful in certain reactions. Optimization of the reaction conditions and high dosage of enzyme may increase the product yield.

Chapter 10
Summary and Conclusion

10.1 Summary

Esterases/lipases are hydrolases acting on acylglycerides and they hold tremendous potential for bio-industrial exploitation. They are among the most used enzymes in industry due to their stability in organic solvents and their ability to catalyze a wide range of reactions with high regio and stereo specificity combined with broad substrate specificity. Considering the ever increasing demand on novel esterase activities, especially for applications which do not have an enzymatic route, it becomes evident that any quest for novel esterases is largely welcome. In this context a study was undertaken to explore cultivable and uncultivable biodiversity for novel esterases/lipases.

A screening of the microbial isolates at CSIR-NIIST identified a novel yeast strain secreting an alkali active, thermostable, halotolerant and solvent tolerant lipase. The culture was identified by morphological and molecular methods to belong to the genera *Pseudozyma*. A two step statistical design of experiment (DOE) methodology was used to improve enzyme production by the novel isolate. The factors affecting lipase production were screened by a two level factorial design and the optimal conditions for lipase production were determined by response surface methodology. The optimization of production conditions resulted in a 9 fold increase in esterase production. The partially purified esterase preparation maintained 90% of its original activity after being treated at a temperature of 70 °C for 1 hr. The partially purified esterase preparation had optimum activity at 60°C and an optimum pH of 8.0, and it retained 97 % of its original activity in presence of 25% methanol and 80% activity in the same strength of ethanol. The enzyme was also active in 2.0 M NaCl.

Zymogram analyses of the crude extract revealed the presence of 3 isoforms in the enzyme preparation. One of the isoforms – *'LIP1'* was purified to homogeneity through hydrophobic interaction chromatography (HIC) and characterized. The purified *LIP1* had a *K*m and V_{max} of 0.01mM and 1.12 mM min^{-1} respectively. *LIP1* was analyzed for its solvent, temperature and salt tolerance. The purified esterase was found to lose its thermo and halo tolerance, but interestingly retained 97 % activity in methanol, a polar organic solvent. The properties exhibited by the crude enzyme could have been

contributed by the isoforms other than *LIP1* and this explains the reason for disparity in enzyme properties between the crude and purified esterase preparations. The purified esterase on SDS-PAGE showed a single band of molecular weight 175 kDa.

Since the possibility of obtaining lipases/esterases with novel properties is more from the unexplored non-cultivable microbial diversity, a metagenomic approach was used to screen for novel esterase activities. Environmental DNA was isolated from Western Ghats (Silent Valley National Park) soil and a soap factory effluent sludge; and after purification was used to construct metagenomic libraries in Fosmid and Bacterial Artificial Chromosome (BAC) vectors. Recombinant clones from Fosmid library (32011) and BAC libraries (1369) were screened for esterases, out of which 21 were positive for esterases. Among the 21, two clones were from silent valley soil metagenome and the remaining 19 were from the effluent sludge metagenome. The occurrence of esterase producing clones in the metagenomic libraries was very high indicating an exceptionally high occurrence of lipolytic organisms in the soap factory effluent stream.

Crude esterases were analyzed for novelty by finding the specificities for fatty acid chain length, tolerance to organic solvents, temperature and salinity. Enzymes from many of the positive clones had important properties like high thermo stability and tolerance towards solvents and high salt concentrations, which are particularly useful to the industry. Esterase from clones 1(41) and 9(62) were temperature stable (80 ^{o}C), solvent tolerant (up to 25 % methanol) and halo tolerant (up to 2M NaCl) and these properties make them very unique and highly desirable for industrial applications including transesterification reactions for biodiesel production; and in organic synthesis. Esterases obtained from the Silent valley metagenomic clones 6(11) and 6(12) were found to be thermo tolerant and solvent tolerant but the esterase activity was significantly less compared to other esterases. Majority of the esterases obtained from metagenomic library can be included under carboxyl esterases (EC 3.1.1.1) rather than lipases (EC 3.1.1.3) based on the substrate specificity and chain length preferences they have shown. None of the positive clones showed activity with esters of long chain fatty acids indicating that the positive clones harbored non-specific esterases and not lipases.

Micro-diversity analyses on esterase positive clones were performed using 16S rRNA universal primers 27F and 1492R. On BLAST analysis 16S rRNA amplicon of clone 6 (11) and 6(12) showed 96% identity with *Paenibacillus* sps. Diversity analyses from the insert of the clones 1(40), 5(1) and 5(4) revealed homology with uncultured

bacteria indicating the novelty of the obtained esterases, which is the primary purpose in adopting a metagenomics approach.

Purification, characterization and cloning of potent esterase *EST1* from effluent metagenome clone 9(62) was carried out. A two step purification strategy using ammonium sulfate precipitation and ion exchange chromatography was performed to purify *EST1* from clone 9(62). Purified esterase had reduced tolerance to salt and solvents. The radical activity loss observed may be due to the conformational change occurred during stringent purification conditions. Molecular weight of the esterase was analyzed through non reducing SDS-PAGE and calculated as ~ 40 kD. Partial amplification of the esterase gene in selected lipase positive clones - 9(62) and 6(11) were performed using degenerate primers designed based on oxyanion hole and active site signature of lipase. On BLAST analysis, the sequence from clone 9(62) showed significant similarity with hypothetical proteins from *Acinetobacter* strains annoted as carboxyl esterases. The hits also possessed the α,β hydrolase fold suggesting hydrolase activity and which is a common occurrence in esterases. BLAST analysis of the sequence from clone 6(11) showed 70% similarity to GDSL hydrolase which is a diverse family of lipases or esterases of *Paenibacillus* sp. Distantly related members of the α-β hydrolase super family share similar conserved motifs with the lipases/esterases in the oxyanion hole and active site, thus making these genes targets for gene prospecting by PCR.

Application of the novel esterases in synthesis and transesterification reactions were evaluated using the enzyme immobilized on magnetic nano particles (MNP). APTES modified magnetite particles functionalized with glutaraldehyde were used to covalently immobilize the esterases and these MNPs were used for production of the flavor compound- ethyl acetate. MNP immobilized *Pseudozyma* esterase could successfully catalyze the synthesis of ethyl acetate and a steady increase in the ethyl acetate concentration was observed with increase in the duration of incubation. The amount of ethyl acetate produced after 24, 48 and 72 h of incubation were 6.24, 8.17 and 12.82 mM respectively. Transesterification studies were also conducted successfully using the esterase immobilized MNPs with vegetable oil as substrate for production of Fatty acid methyl esters (FAME/ Biodiesel). The immobilized enzymes retained 100% activity even after storage for 3 months at 4 °C. This ensured the stability and scope in reusing immobilized magnetic nanoparticles for biotransformation reactions.

10.2. Conclusion

Studies were undertaken to explore cultivable and uncultivable biodiversity for novel esterases/lipases through classical and metagenomic approach. Search for novel esterases through classical approach yielded new strain of *Pseudozyma*, whereas the metagenomic approach provided several clones producing esterases with certain interesting properties. Among the metagenomic clones, the esterase *EST1* from clone 9(62) was interesting due to its tolerance for temperature, solvent and salt. However, the purified *EST1* esterase did not retain the tolerances towards temperature, solvent and salt.

While the purified *Pseudozyma* esterase (*LIP1*) lost its thermo and halo tolerance, it was still active at 25% solvent. For all practical industrial applications requiring thermo, halo or solvent tolerances, highly pure enzymes may not be a must, and this make a case for the crude *Pseudozyma s*p esterase. The concept of its application in synthesis and transesterification and its easy recoverability and reusability was demonstrated by the magnetic nanoparticle experiments where these capabilities were proved. The present works shall serve as platform to continue efforts in developing this enzyme immobilized catalyst further, for commercial applications.

The untapped microbial biodiversity of uncultivable microbes is a rich resource to prospect for novel bioactivities. This was proved again with this study where the libraries constructed from the exotic western ghat soil as well as sludge from a soap factory effluent both yielded esterases with several interesting properties. The unusually high occurrence of lipase positive clones from the effluent sludge metagenome confirms that the choice of starting material for esterase producing microbes was correct. The esterase positive clones displayed diverse and as yet undescribed lineages since the matches to their 16s rDNA were mostly uncultured bacteria. This is interesting since it opens up the possibilities of having esterases which may be different from the known types.

The libraries from both silent valley soil DNA and from the effluent sludge DNA could also be a treasure trove for other bioactivities which can be explored further. It may be concluded that the prospecting for esterases by classical and metagenomic approach had been successful with potent esterases obtained from both the strategies. It would be prudent to look further into the complete gene sequence and protein structure of the novel esterases described here which could be logical follow up of the work.

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Chapter 1

Introduction and Review of Literature

Chapter 2

Materials and Methods

Chapter 3

Screening and selection of a novel microbe for esterase production

Chapter 4

Fermentative production of esterase from *Pseudozyma* sp NII 08165

Chapter 5

Purification and characterization of esterase from *Pseudozyma* sp NII08165

Chapter 6

Construction and screening of metagenomic libraries for novel lipases

Chapter 7

Studies on the properties of esterases from metagenomic clones

Chapter 8

Purification and characterization of esterase from metagenomic library clone and cloning of the partial gene sequences.

Chapter 9

Studies on magnetic nanoparticle immobilized esterase for biotransformation reactions.

Chapter 10
Summary and Conclusion

APPENDIX 1

LIST OF ABBREVIATIONS

#	Number
%	Percentage
℃	Degree Celsius
µ	Micron/ micrometer
µg	microgram
µl	microlitre
ANOVA	Analysis of Variance
BAC	Bacterial Artificial Chromosoma
BLAST	Basic Local Alignment Search Tool
bp	base pair
CODEHOP	COnsensus DEgenerate Hybrid Oligonucleotide Primers
CSIR	Council of Scientific and Industrial Research
CTAB	Cetyl Trimethyl Ammonium Bromide
Da	Dalton
DNA	Deoxy Ribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DOE	Design of Experiments
EDTA	Ethylene Diamine Tetra Acetic Acid
FID	Flame Ionization Detector
Fig	Figure
FTIR	Fourier transform infrared spectroscopy
g	gram
g/g	gram per gram
g/L	gram per litre
GC	Gas chromatography
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIC	Hydrophobic Interaction Chromatography
ITS	Internal Transcribed Spacer Region
IU	International Units
kD	kilo Dalton
Km	Michaelis constant
L/l	Liter
lbs	Pounds Inch $^{-2}$
M	Molar
mg	milligram
min	minute

ml	milliliter
mM	millimolar
MNP	Magnetic Nano Particle
MUB	Methylumbelliferyl Butyrate
NII	NIIST Culture Collection ID
NIIST	National Institute for Interdisciplinary Science and Technology
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
pNP	para Nitro Phenol
pNPB	para Nitro Phenyl Butyrate
pNPL	para Nitro Phenyl Laurate
pNPM	para Nitro Phenyl Myristate
pNPO	para Nitro Phenyl Octanoate
pNPP	para Nitro Phenyl Palmitate
pNPV	para Nitro Phenyl Valerate
RNA	Ribonucleic Acid
rpm	Rotations per minute
rRNA	Ribosomal RNA
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscope
SmF	Submerged Fermentation
sp	Species
Taq	Thermus aquaticus
TBR	Tributyrin agar
U	Units
U/ml	Units/milliliter
UV	Ultra Violet
v/v	Volume /Volume
Vmax	Maximum Velocity
w/v	Weight/Volume
YPD	Yeast Extract Peptone Dextrose