"BIOTRANSFORMATION: IN SYNTHESIS OF FATTY ACID ALKYL ESTERS FROM *JATROPHA* AND *PONGAMIA* OIL"

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BY

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DECLARATION BY RESEARCH GUIDE

This is to certify that the work incorporated in the thesis entitled, "**Biotransformation: In synthesis of fatty acid alkyl esters from Jatropha and Pongamia oil",** submitted by **Ms. Jayshree B. Kantak**, for the Degree of ***Doctor of Philosophy***, was carried out by the candidate under my supervision at Division of Biochemical Sciences, National Chemical Laboratory, Pune-411008, India. Material that has been obtained from other sources is duly acknowledged.

Dr. (Mrs). Asmita. A. Prabhune

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DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled "**Biotransformation: In synthesis of fatty acid alkyl esters from Jatropha and Pongamia oil"**, submitted for the Degree of **Doctor of Philosophy** to the University of Pune, has been carried out by me under the supervision of Dr. Asmita. A. Prabhune at Division of Biochemical Sciences, National Chemical Laboratory, Pune 411008, India. This work is original and has not been submitted in part or full by me for any other degree or diploma to any other University.

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..... Dedicated to My Dear

Mother

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Chapter I

General Introduction

1.1. Introduction:

In recent years, the whole world has been challenged by two different crises about fossil fuel, its depletion and environmental degradation due to their prolonged use. The indiscriminate exploration and consumption of fossil fuels has led to a reduction in petroleum reserves and also environmental consequences of exhaust gases from petroleum fuelled engines. The continuous use of petroleum has intensified problems of air pollutants and carcinogens [Ma and Hanna, 1999]. To protecting the global environment and fulfil the long-term supplies of petro-diesel fuels, it's becoming necessary to develop alternative fuels which are comparable with conventional fuels. Alternative fuels should be, not only sustainable but also environment friendly. Fuels of biological origin, such as alcohol, vegetable oils, biomass, biogas, synthetic fuels etc. are becoming important. Along with these fatty acid alkyl esters of vegetable oils and animal fats has received considerable attention since several years. Alkyl esters of fatty acids are called as biodiesel, biodiesel are renewable, biodegradable, and nontoxic fuel [Fukuda et. al., 2001]. Biodiesel is a renewable green alternative to the petro-diesel due to its total combustion property [Ma. and Hanna, 1999]. One hundred years ago, Rudolf Diesel tested vegetable oil as fuel for his engine (Shay, 1993). With the advent of cheap

petroleum, appropriate crude oil fractions were refined to serve as fuel. . In the 1930s and 1940s vegetable oils were used as diesel fuels from time to time only in emergency situations. The direct use of vegetable oils and/or oil blends is generally considered to be unsatisfactory and impractical for both direct-injection and indirect type diesel engines. Ma and Hanna in, 1999 have discussed the problems like non atomization of the injector which leads to further problem like carbon deposits, oil ring sticking and thickening and gelling of the lubricating oil as a result of contamination by the vegetable oils. Different pre-treatments were reported for reducing the high viscosity of vegetable oils [Ma and Hanna, 1999] which are discussed briefly here as follows.

1.5. Methods of pre-treatments for vegetable oils:

(a) Dilution (Blending)- Dilution of oil with solvents to lower viscosity. Dilution of vegetable oils can be accomplished with materials such as diesel fuels, or solvent like ethanol. Dilution reduces the viscosity, engine performance problems such as injector chocking and more carbon deposits.

(b) Microemulsion- Microemulsions (vegetable oils, esters, and co-solvents such as short chain alcohols, as dispersing agents) to lower viscosity. A microemulsion can be made of vegetable oils with an ester and dispersant (co-solvent), or of vegetable oils, an alcohol and a surfactant, with or without diesel fuels. Ziejewski et al. in, 1984 reported several problems on use of microemulsification consisting of oil and solvent such as irregular injector needle sticking, heavy carbon deposits, incomplete combustion and an increase of lubricating oil viscosity.

(c) Pyrolysis- The pyrolysis of fat (thermal decomposition at high temperatures to reduce the molecular size for use as biodiesel) to lower viscosity. The pyrolysis of vegetable oil was conducted in an attempt to synthesize petroleum from vegetable oil. Since World War I, many investigators have studied the pyrolysis of vegetable oils to obtain products suiTable for fuel. In 1947, a large scale of thermal cracking of tung oil tung oil (China wood oil) calcium soaps was reported (Wan, 1947).

(d) Catalytic cracking- Catalytic cracking of vegetable oils to produce biofuel has been studied (Pioch et. al., 1993). In this study they reported, that the process was simple and effective compared with other cracking processes. There was no waste water or air pollution by using this catalytic cracking process.

(e) Transesterification- This procedure is based on conversion of short-chain alcohols to form lower-chain fatty acid alkyl esters (FAAE). Due to this process oil becomes volatile and less viscous, although viscosity depends on fatty acid composition. This process is described in detail in following section.

1.6. Transesterification of oil:

Transesterification appears to be the simplest and the best route to produce biodiesel, in large quantity. Physical characteristics of fatty acid esters (biodiesel) are very close to those of fossil diesel fuel with little or no deposit formation after combustion in diesel engines and the process is relatively simple [Ma and Hanna, 1999]. Biodiesel has been produced from a variety of edible vegetable oil sources such as soybean, sunflower, cottonseed, rapeseed and palm oil.

Transesterification is the general term used to describe the important class of organic reactions where an ester is transformed into another ester through interchange of the alkoxy moiety (Figure 1.1). When the original ester is reacted with an alcohol, the transesterification process is called alcoholysis [Otera, 1993]. The transesterification is an equilibrium reaction, and the transformation occurs essentially by mixing the reactants. However, the presence of a catalyst (typically a strong acid or base) accelerates attainment of equilibrium. In order to achieve a high yield of the ester, the alcohol has to be used in excess.

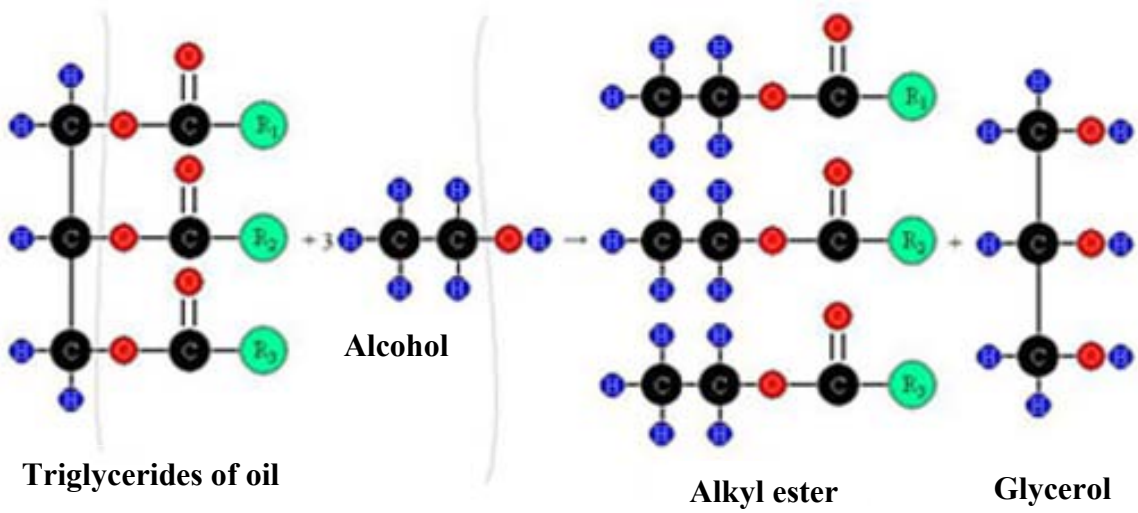


Figure.1.1. Biodiesel production by alcoholysis with short chain alcohols

Various approaches with respect to catalyst have been used for transesterification of oil with alcohol. Which includes Chemical catalysis with acids or alkalis, enzymatic catalysis which uses lipase enzyme as catalyst and whole cells of lipase producing organisms as catalyst.

1.7. Methods of Transesterification

1.7.1. Chemical Catalysis:

In chemical catalysis, alcoholysis of oil by methyl or ethyl alcohol takes place in the presence of a strong acid or base which produces biodiesel and glycerol. Chemical ways have realized the industrialization of biodiesel in many countries. It usually includes two kinds of catalyst: alkali catalysts and acid catalysts (Ma and Hanna 1999).

1.7.1.1. Alkali catalyst:

Alkali-catalyzed transesterification is much faster and is most often used commercially. The alcohol–oil molar ratio that should be used varies from 1:1–6:1. The types of alcohol are usually methanol and ethanol. Ethanol has fewer safety problems because it is less toxic [Marchetti; et. al., 2007]. The alkalis include NaOH, KOH, carbonates and corresponding sodium and potassium alkoxides such as sodium methoxide, sodium ethoxide, sodium propoxide and sodium butoxide. The amount of catalyst that should be added to the reactor varies from 0.5% to 1% w/w [Barnwal and Sharma, 2005], but in some reports it was mentioned the values between 0.005% and 0.35% w/w [Ma and Hanna, 1999]. Temperature remains an important variable in all these reactions. The standard value for the reaction to take place is 60°C, but depending on the type of catalyst different temperatures will give different degrees of conversion [Zhang, et. al., 2003; Noureddini and Zhu, 1997; Mittelbach and Tratnigg ,1990]. Low free fatty acid content in triglycerides is required for alkali-catalyzed transesterification which is reported by Wright et. al., in 1944. In case of refined oil, alkali process always gives high conversion, and the reaction rate is reasonably high even at a low temperature of 60°C. For an alkali-catalyzed transesterification, the glycerides and alcohol must be substantially anhydrous. However, if the oil has high free fatty acid and/or water content, soap formation is likely to take place. Saponification not only consumes the alkali catalyst but also the resulting soaps can cause the formation of emulsions and gels,

which creates difficulties in downstream recovery of glycerol and purification of the biodiesel (Ma and Hanna 1999). Sodium methoxide has been found to be more effective than sodium hydroxide, presumably because a small amount of water is produced upon mixing NaOH and MeOH (Freedman, et. al., 1984; Hartman, 1956]. However, sodium hydroxide and potassium hydroxide are also able to catalyze transesterification, and because of their low cost, are widely used in industrial biodiesel production [Nye, et. al., 1983].

1.7.1.2. Acid catalyst:

Acid-catalyzed transesterification is suitable for feedstock with high free fatty acid or water content. Commonly used acid catalysts are sulfuric acid, sulfonic acids and hydrochloric acid (Keim, 1945). In the acid reaction, if an excess of alcohol is used in the experiment then better conversion of triglycerides is obtained, but recovering glycerol becomes more difficult. Hence optimal relation between alcohol and raw material should be determined experimentally considering each process [Marchetti, et. al., 2007]. Freedman and Pryde in 1984 got the desirable product with 1 mol% of sulfuric acid with a molar ratio 30:1 of ethanol to oil at 65 °C with 99% of conversion in 50 h, whereas the butanolysis needed will need 117 °C and the ethanolysis 78 °C but the times should be 3 and 18 h, respectively. In comparison with alkali process, the conversion using acid process is high, while the acids, being corrosive, may cause damage to the equipment. Also the reaction rates by acid catalysis for converting triglycerides to methyl esters are slow. Acid-catalyzed reactions require higher reaction temperatures (100°C) and longer reaction times than alkali-catalyzed transesterification.

1.7.2. Biological Catalysis:

Chemical transesterification is efficient in terms of reaction time; however, the chemical approach to synthesize biodiesel from triglyceride has drawbacks, such as difficulty in the recovery of glycerol and the energy-intensive nature of the process. In contrast, biocatalysts allow synthesis of specific alkyl esters, easy recovery of glycerol, and transesterification of glycerides with high free fatty acid content [Nelson, et. al., **1996]**. New biochemical routes to biodiesel production using enzymes are becoming more attractive [Chang, et. al., 2005; Lai, et. al., 2005; Fukuda, et. al., 2001]. In recent years, enzyme / whole cells for biodiesel production have drawn an increasing attention. As compared to chemical approaches, lipase-mediated alcoholysis for biodiesel production has many advantages [Table 1.1]. The possibility of using lipase enzymes in organic solvents has opened up several exciting avenues for biotransformation, due to its stability in organic solvents [Clapes, et. al., 1995, Gupta, 2000].

1.7.2.1. Enzymes as catalyst:

An alternative approach to biodiesel production is the use of naturally occurring enzymes (lipases (EC 3.1.1.3) to catalyze the reaction. Nelson et al., in 1996 investigated the ability of lipases for transesterification of triglycerides with short-chain alcohols to produce alkyl esters. Such an approach promises several advantages over the conventional approach to biodiesel production using chemical processes. Nelson et al. in 1996 reported use of different feed stocks such as yellow grease, brown grease and other degraded, low-cost oils for biodiesel production using enzyme as catalyst. This eliminated soap production under high FFA conditions and the presence of FFAs in the feedstock increased the yield. If enzymatic catalysis becomes sufficiently inexpensive, it could even be applied to virgin vegetable oils since it requires less energy (reaction

temperatures are typically less than 37◦C), less alcohol and produces fewer wastes material and it works even in the presence of water [Haas, et. al., 2002].

Table 1.1. Comparison between alkali-catalysis and lipase-catalysis methods for biodiesel fuel production

	Alkali- Catalyzed	Lipase- Catalyzed
Reaction temperature	60-70°C	30-40°C
Free fatty acids in raw materials	Saponified products	Methyl esters
Water in raw materials	Interference with the reaction	No influence
Yield of methyl esters	Normal	Higher
Recovery of glycerol	Difficult	Easy
Purification of methyl esters	Repeated washing	None
Production cost of catalyst incurring	Cost effective	Cost

Lipases belong to a group of hydrolytic enzymes called hydrolases. In biological systems, lipases hydrolyze triglycerides (fats) to fatty acids and glycerol. Lipases are classified as those esterases that can hydrolyze long chain acyl glycerols [Verger, 1997].

Lipases have some unique properties which make them most widely used group of biocatalysts in organic chemistry. The first important factor is, they usually display exquisite chemoselectivity, regioselectivity and stereoselectivity, they are readily available in large quantities because many of them can be produced in high yields from microbial organisms, namely fungi and bacteria. Also the crystal structures of many lipases have been solved, facilitating considerably the design of rational engineering strategies. And the main advantage is they do not usually require cofactors nor do they catalyse side reactions. This is reflected not only by many original articles on lipases that appear each year, but also by number of excellent reviews covering this topic [Fukuda, et. al., 2001; Ma and Hanna , 1999; Kaieda, et. al., 2001]

Lipases, have a specific, active three dimensional structure in an aqueous environment with polar groups exposed and non-polar groups buried inside (Casimir, et. al. 2007). A lipase is an enzyme that catalyzes the formation or cleavage (hydrolysis) of fats (lipids). Lipases are a subclass of the esterases. Lipases are very important in the digestion of lipids. These enzymes perform essential roles in the digestion, transport and processing of dietary lipids (e.g. triglycerides, fats, oils) in most, if not all, living organisms. Genes encoding lipases are even present in certain viruses.

Unlike other enzymes, the nature of a lipolytic reaction catalyzed by lipases is very complex, in which the lipid substrates are water-insoluble [Sellappan and Akoh, 2005]. The need for some water to maintain and activate lipase and the immiscibility of lipids in water make the reaction media heterogeneous by forming a liquid–liquid interface. The interface is the point where the lipase can access the substrate and catalyze the reaction. The nature of the interface, interfacial properties, and interfacial area affect the enzyme action. The interface activates the enzyme by adsorption, which aids the opening of the lid on the catalytic site [Shaw, et. al., 1990]. All types of interfaces, such as solid–

liquid, liquid–liquid, or liquid–gas, can influence the activity due to the interfacial hydrophobicity. Both intra- and extracellular lipases are designed to catalyze hydrolytic reactions since the living cells are made up of and surrounded by a water-rich environment. Water plays an important role as a medium to disperse the enzyme molecule and participates as a co-substrate in hydrolysis [Sellappan and Akoh, 2005].

1.7.2.2. Applications of Lipases:

Lipases constitute the most important group of biocatalysts for biotechnological applications. Along with the most upcoming and interesting application in biodiesel industry, lipases are known to have many more important commercial applications which are listed in the **Table 1.2** and described below.

Table 1.2. Applications of lipase

Application	**Examples**	**Reference**
Detergents	Detergents such as Lumafast from *Pseudomonas mendocina* and Lipomax from *Pseudomonas alcaligenes*	Houde , et. al. 2004
New biopolymeric materials	Trimethylolpropane esters were also similarly synthesized as lubricants	Linko , et. Al. 1998
Synthesis of fine chemicals: *Therapeutics* *Agrochemicals* *Cosmetics and flavours*	*Pseudomonas* AK lipase in synthesise the chiral intermediate in the total synthesis of the potent antitumour agent epothilone enantiopure (*S*)-indanofan, a novel herbicide used against grass weeds in paddy fields	Zhu and Panek, 2001
Food	Polyunsaturated fatty acids (PUFAs) play an	Gill and

ingredients	increasingly important role as nutraceutical agents	Valivety, 1997
Lipase in textile industry	desizing of denim and other cotton fabrics. improves dyeing, ability of cloth to uptake chemical compounds	Fariha Hasan et. al. 2006
Diagnostic tool	diagnostic tool for detecting acute pancreatitis and pancreatic injury Characterizing the virulence factors of *Aeromonas* bacteria from municipally treated drinking	Lott and Lu, 1991 Sen ,et. al., 2004
Pulp and paper industry	In removing the pitch (the hydrophobic components of wood, namely triglycerides and waxes) from pulp produced in the paper industry.	Fariha Hasan, 2006
Degreasing of leather	Degreasing of fatty raw materials such as small animal skins and hides	Fariha Hasan, 2006
Medical applications	Lipases from the wax moth (Galleria mellonella) were found to have a bacteriocidal action on *Mycobacterium tuberculosis* H37Rv	Annenkov , 2004
Lipases as biosensors	Immobilized lipases on electrodes function as lipid biosensors and may be used in triglycerides and blood cholesterol determinations	Fariha Hasan, et.al., 2006
Biodiesel production	Various lipases listed in Table 2.2	

i) Detergents

The most commercially important field of application for hydrolytic lipases is their addition to detergents, which are used mainly in household, industrial laundry and in household dishwashers. To improve detergency, modern types of heavy duty powder

detergents and automatic dishwasher detergents usually contain one or more enzymes, such as protease, amylase, cellulase and lipase [Ito, et.al. 1998]. Enzymes can reduce the environmental load of detergent products, since they save energy by enabling a lower wash temperature to be used, are biodegradable, with no harmful residues, have no regarding effluent treatments, no risk to aquatic life. In 1994, Novo Nordisk introduced the first commercial lipase, Lipolase™, which originated from the fungus *T. lanuginosus* and was expressed in *Aspergillus oryzae.*

ii) New biopolymeric materials:

Lipases and esterases are used as catalysts for polymeric synthesis with the major advantages being their high selectivity (e.g. stereoselectivity, regioselectivity and chemoselectivity) under mild reaction conditions. Biopolymers like polyphenols, polysaccharides and polyesters show a considerable degree of diversity and complexity because they are biodegradable and are produced from renewable natural resources. Structurally complex monomers with multifunctional reactive groups were polymerised in a high-throughput enzymatic catalysis using commercially available lipases from different sources.

iii) Synthesis of fine chemicals:

Lipases perform the role of intermediates in the synthesis of therapeutics, agrochemicals and flavour compounds which are difficult to synthesise with chemical methods.

a. Therapeutics

Several new examples of lipase-catalysed enantioselective reactions for the synthesis of pharmaceuticals are available. *Pseudomonas AK* lipase was used to synthesise the chiral intermediate in the synthesis of the potent antitumour agent epothilone. Lipase from *Candida rugosa* catalysed the enzymatic resolution of the antimicrobial compounds (S)- and (R)-elvirol and their derivatives (S)-(+)- and (R)-(–)-curcuphenol [Karl-Erich Jaeger and Thorsten Eggert, 2002].

b.Agrochemicals Lipases are used in the production of different herbisides and insecticides. The examples include production of (S)-indanofan, a novel herbicide used against grass weeds in paddy fields [Karl-Erich Jaeger and Thorsten Eggert, 2002].

c.Cosmetics and flavours

Lipase assists in synthesis of different flavour and fragrance compounds. The best example is *Burkholderia cepacia* lipase which was used in the production of menthyl methacrylate 16 which is polymerised and used as a sustained release perfume. The plant growth factor (–)-methyl jasmonate is another important perfume constituent, which can be synthesised using the commercially available Lipase P [Karl-Erich Jaeger and Thorsten Eggert, 2002].

iv) Food ingredients

Cocoa butter contains palmitic and stearic acids and has a melting point of approximately 37°C, leading to its melting in the mouth, which results in a perceived cooling sensation. Immobilized lipase can be used in synthesis reaction to produce a cocoa-butter. Polyunsaturated fatty acids (PUFAs) play an increasingly important role as nutraceutical agents [Gill and Valivety, 1997]. These are from the class of essential fatty acids, which are required for membrane-lipid and prostaglandin synthesis. Microbial

lipases are used to enrich PUFAs from animal and plant lipids, such as menhaden, tuna or borage oil.

v) Textile industry

A commercial preparations of Lipases and alpha amylases helps in desizing of denim and other cotton fabrics. Lipases along with other enzymes also used to increase the absorption property of fabric which improves dyeing, it is also used to reduce the streaks and cracks in the denim abrasion systems [Shaw, et. al.,1990]. In the textile industry, synthetic fibres such as polyesters have been modified enzymatically for the use in the production of yarns, fabrics, rugs and other consumer items. Lipases helps in post-modification treatments and witch improves the ability of cloth to uptake chemical compounds, such as cationic compounds, fabric finishing compositions, dyes, antistatic compounds, anti-staining compounds, antimicrobial compounds, antiperspirant compounds and/or deodorant compounds [Hasan et. al., 2006].

vi) Degreasing of leather

It is well described by Hasan, et. al., 2006 a well known thing about lipases is they can degrade the fats, and fat removal is the main hurdle which was solved by the lipases. The best example is for sheepskins, which contain up to 40% fat, the use of solvents is very common and these can also be replaced with lipases and surfactants. Lipase enzymes can remove fats and grease from skins and hides, particularly those with a moderate fat content. Both alkaline stable and acid active lipases can be used in skin and hide degreasing.

vii) Pulp and paper industry

Another application field of increasing importance is the use of lipases in removing the pitch (the hydrophobic components of wood, namely triglycerides and waxes) from pulp produced in the paper industry. Pitch cause severe problems in pulp and paper manufacture. Nippon Paper Industries in Japan developed a pitch control method that uses a fungal lipase from *C. rugosa* to hydrolyse up to 90% of the triglycerides [Fariha Hasan, 2006].

viii) Diagnostic tool

Lipases are also important drug targets or marker enzymes in the medical sector. They can be used as diagnostic tools and their presence or increasing levels can indicate certain infection or disease. The level of lipases in blood serum can be used as a diagnostic tool for detecting conditions such as acute pancreatitis and pancreatic injury [Lott and Lu, 1991].

Characterizing the virulence factors of *Aeromonas* bacteria f municipally treated drinking water is a source of potentially pathogenic *Aeromonas* bacteria [Sen and Rodgers, 2004].

ix) Medical applications

Lipases are the activators of Tumor Necrosis Factor and therefore can be used in the treatment of malignant tumours [Kato, 1989]. Lipases have earlier been used as therapeutics in the treatment of gastrointestinal disturbances, dyspepsias, cutaneous manifestations of digestive allergies, etc. [Mauvernay, et. al, 1970]. Free PUFAs and their mono- and diglycerides are subsequently used to produce a variety of pharmaceuticals including anticholesterolemics, anti-inflammatories and thrombolytics [Gill and Valivety, 1997].

x) Lipases as biosensors

Lipases may be immobilized onto pH/oxygen electrodes in combination with glucose oxidase, and these function as lipid biosensors and may be used in triglycerides and blood cholesterol determinations [Hasan, et.al., 2006]. Immobilized lipases on electrodes function as biosensor based on the enzyme-catalysed dissolution of biodegradable polymer films has been developed.

xi) Biodiesel production:

As the price of petroleum fuel keeps rising everyday and in addition to environment concerns, there is need to research the alternative fuels such as biodiesels. Biodiesels are alkyl esters triglycerides of oil. The commercial process commonly used to produce biodiesel is the chemical process that utilizes alkaline catalysts (sodium or potassium hydroxide) to convert vegetable oil or fat and methanol to fatty acid methyl esters, FAME. However, they form soap, which consumes the catalyst, decreases yield, thereby making purification and isolation of the FAME difficult. Glycerol, a co-product of the chemical alcoholises reaction if removed easily then the processes will be cost effective. But in alkaline catalyzed reactions purification of product and glycerols is very difficult due to interference of soaps. The best alternative to this process is the enzymatic alcoholises of fats and oils. A number of lipases have been explored as catalysts in biodiesel production **(Table 1.3).**

Table 1.3. Enzymatic transesterification reactions using various types of alcohols and lipases

Oil	Alcohol	Lipase	Conversion	Solvent	Ref.
Rapeseed	2-Ethyl-1-Hexanol	*C. rugosa*	97	None	**Linko,et. al. 1998**
Mowrah,	C_4-$C_{18.1}$				
Mango, Kernel, Sal	alcohols	*M. Miehi* (Lipozyme IM 20)	86.8-99.2	None	**De, et.al., 1999**
Sunflower	Ethanol	*M. Miehi* (Lipozyme)	83	None	**Selmi, et. al. 1998**
Fish	Ethanol	*C. antartica*	100	None	**Breivik,et. al., 1997**
Recycled restaurant Grese	Ethanol	*P.cepacia* (LipasePS-30) + (*C. antratica* SP-435)	85.4	None	**Wu, et. al., 1999**
Tallo,	Primary alcohol[a]	*M. Miehi* (Lipozyme IM60)	94.8-98.5	Hexane	**Nelson, et. al., 1996**
soyben	secondary, alcohol[b]	*C. antratica* (SP-435)	61.2-83.8	Hexane	
Rapeseed	Methanol, Ethanol	*M. Miehi* (Lipozyme IM60)	19.4, 65	None	
Sunflower	Methanol	*P.fluorescens*	79	Petroleum ether	**Mittelbach, 1990**
Ethanol			82	None	

Palm kernel	Methanol	*P.cepacia* (LipasePS-30)	15	None	**Abigor,et. al., 2000**
Ethanol			72	None	
Jatropha oil	Ethanol	*Chromobacterium viscosum*	62	n-hexane	Shah, et. al.,2004
Soybean oil	methanol	*C. rugosa* *R. oryzae*	99	None	Lee,et.al.,2006

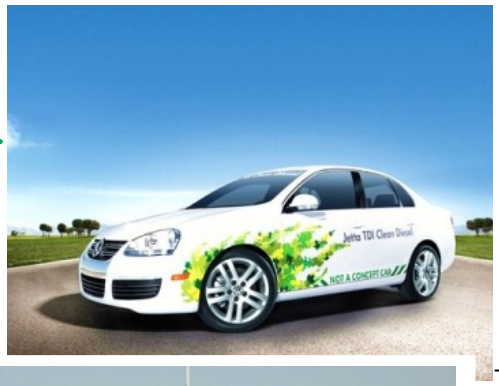
[a] Methanol, ethanol, propanol, butanol, and isobutanol.

[b] Isopropanol and 2-butanol.

A rapid expansion in production capacity is being observed not only in developed countries such as Germany, Italy, France, and the United States but also in developing countries such as India, China, Brazil, Argentina, Indonesia, and Malaysia. Taiwanese researchers achieved a yield of 92% using *Rhizomucor miehei* lipase [Shieh, et. al. 2003] Researchers in China, testing a variety of lipases, achieved yields of up to 94% [Deng, et. al., 2003; Du., et. al., 2004]. Another report from Italy tested several lipases and found that one produced from the bacterium *Pseudomonas cepacia* gave 100% yield in 6 hours.[Salis, et. al., 2005]. Similar work was performed in the United States.[Noureddini, et. al., 2005] . The U.S. Department of Agriculture has investigated wide variety of lipases and found many to be very effective catalysts for biodesiesl production [Abigor, et. al., 2000; Hsu, et. al., 2001]. In almost all cases, reactions occurred at temperatures under 37.7 °C.

In India extensive research is going on transesterification of the non-edible and edible oils such as *Jatropha*, *Pongamia*, *Helianthus annuus* and etc S. Shah et.al, in 2004 reported 92% yield of fatty acid methyl esters from *Jatropha* oil with the help of immobilized *Chromobacterium Viscosum lipase[Shah, et. al., 2004]*. Modi et. al. in

2007 studied transesterification of crude oils of *Jatropha curcas* (*Jatropha*), *Pongamia pinnata* (karanj) and *Helianthus annuus with* Novozym-435 (immobilized *Candida antarctica* lipase B) and found maximum yield of ethyl esters, 91.3%, 90% and 92.7% respectively [Kaieda, et. al., 2001]. Devanesan, et. al., in 2007 studied the transesterification of *Jatropha* oil and methanol with commercial available immobilized enzyme from the source *Pseudomonas fluorescens* MTCC 103 which gave the maximum yield of 72% was observed in this research [Devanesan, et. al., in 2007]. Following are the different type of transportations which are utilizing the Biodiesel.



Volkswagen, introduced its Clean Diesel Technology on the race track in 2008, announced it's using B5 biodiesel to power its race cars, transport vehicles

February 26, 2009

In the San Francisco area, Red and White Fleet uses biodiesel from Golden Gate Petroleum in its ships.

October 13, 2006

The five new heavy duty transit vehicles will use a combination of soy biodiesel fuel.

April 8, 2010

BioJet 1—up to 17,000 feet (5,180 meters) over 37 minutes. A three minute, 15-second test.

November 30, 2007

Utilization of Biodiesel worldwide in all type of transportations

Unfortunately, lipases are tend to be costly due to purification procedures which makes the process uneconomical [Noureddini, et. al., 2005]. To extend the life of the enzyme, thus reducing its cost, a number of researchers have "immobilized" the lipase in or on a physical structure to stabilize the enzyme and allow its reuses. Research suggests that the most effective immobilization technique is the use of a silica gel, greatly extending lipase life without losing its yield [Hsu, et. al., 2001]. However, construction of the silica gel lipase structure can be time consuming and expensive [Fukuda, et. al. 1996]. In addition, even though reuse of enzyme for several reactions is possible the cost still continues to be very high.

Biocatalysts exhibit advantages over alkali or acid catalysts in that the overall transesterification process is less energy intensive and a complex process of catalyst removal and waste treatment is not required. However, the main hurdle of a traditional biological way of using immobilized lipase as a catalyst is its high cost.

The downstream processing separation, purification, and immobilization of extracellular lipase normally accounts for over 70% of the lipase cost. Thus, the study of using immobilized whole cell catalysts as an alternative to extracellular lipase catalysts has

become a increasingly important, because it can avoid the complex and expensive purification and separation techniques associated with immobilized lipase Ban, et. al., 2001; Zeng, et. al.2006 (10) Ban, et. al., 2002]. It has been demonstrated that *Rhizopus oryzae* whole cell could efficiently catalyze the methanolysis of refined vegetable oils for biodiesel production in a solvent-free system [Fukuda, et. al., 2008; Hama , et. al., 2006]. Shimada et al., in 1999 found that immobilized *C. anturctica* lipase (Novozym 435) was the most effective for methanolysis among lipases tested. In this report stepwise addition of methanol was done to avoid lipase inactivation As a result, more than 95% of the ester conversion was maintained even after 50 cycles of the reaction**.** Watanabe et. al., in 2000 reported an industrially scalable method of methanolysis with Novozym 435 that employed either a batch system consisting of two steps, or a flow reaction of three steps [Fukuda, et. al., 2001]. The methyl esters content in the final elute reached 90–93% and the lipase could be used for a minimum of 100 days in both systems without any significant decrease in yield.

The *R. oryzae* lipase, which exhibited l(3)-regiospecificity, is also effective for the methanolysis of soybean oil [Matori, et. al., 1991; Scheib, et. al., 1998] The lipase efficiently catalyzed methanolysis in the presence of water in the starting material and the enzyme was nearly inactive in the absence of water.

1.7.3. Whole cells as catalyst:

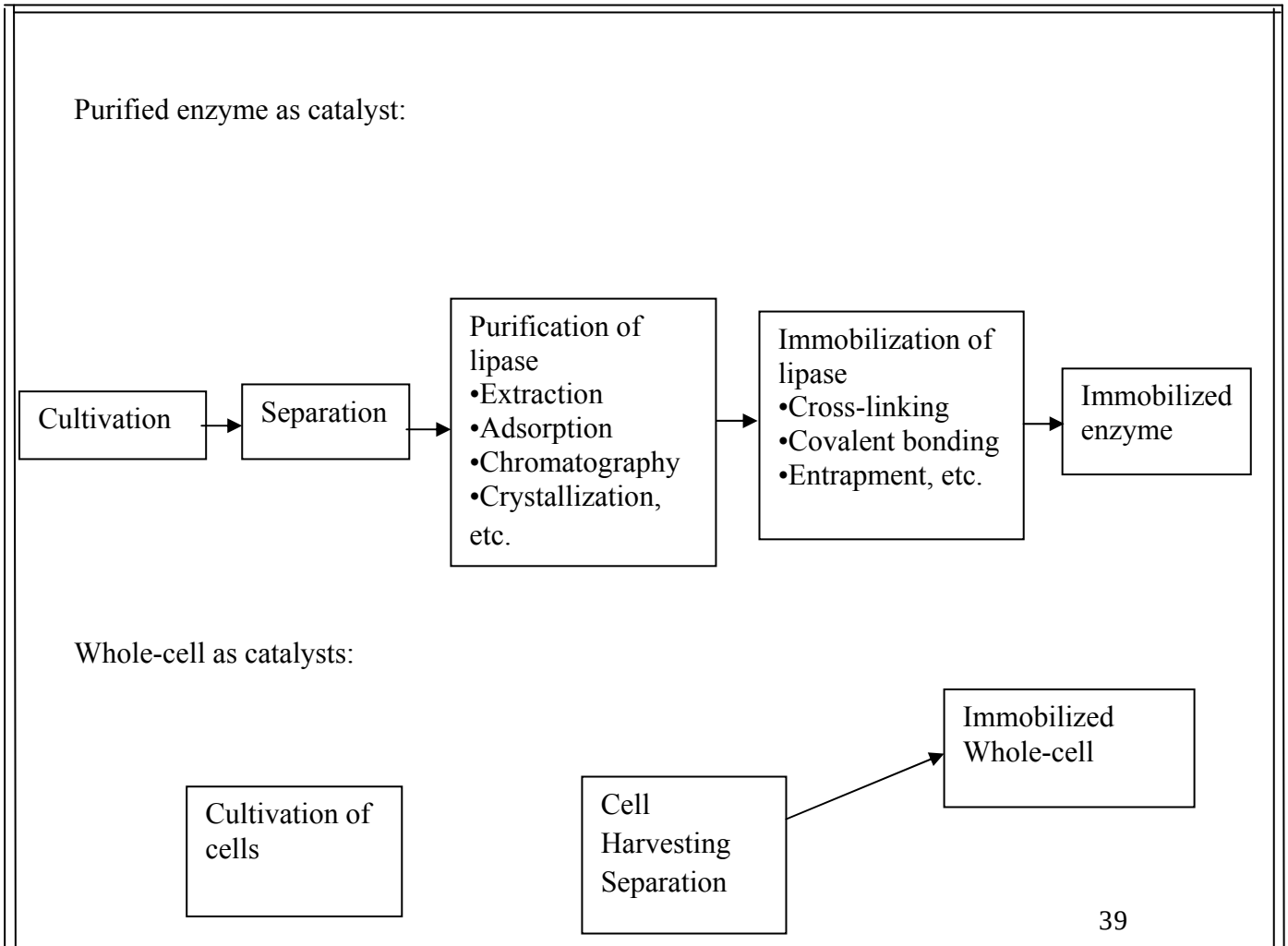
Commercial biodiesel is currently produced by reacting a fat or oil (triglyerides) with methanol in the presence of an alkali catalyst. Although alkali-catalyzed transesterification promotes high conversion rates of triglycerides to their corresponding alkyl esters within short reaction times, the process has several drawbacks. Considering the disadvantages of using chemical catalyst, biological catalysts have their own

importance. Lipases are the enzymes which catalyze transesterification reaction. The cost of lipases significantly limits their application for the bulk production of fuels and chemicals. This prompted researchers into the potential use of microorganisms such as bacteria, yeast and fungi that would serve as whole-cell biocatalysts based on their ability of immobilization and the display of functional proteins of interest on their cell surface. Several studies have reported the utilization of microorganisms such as bacteria, yeast and fungi as whole-cell biocatalysts in attempts to improve the cost effectiveness of the bioconversion processes [Ban et. al., 2001; Fujita et. al., 2002; Narita, et. al., 2006]. All the research focused on the lipase produced by the filamentous fungi *Rhizopus oryzae* whole cell biocatalysts and genetically engineered *S. Cerevisiae* over expressing the *Rhizopus oryzae* lipase production gene [De, et. al., 1999]. The one of the important cost incurring factor in biodiesel fuel production is substrate oil. Tamalampudi, et. al., in 2008 were first to utilize whole-cell catalyzed alcoholysis of the non-edible *Jatropha* oil with *R. oryzae* cells that had been immobilized into BSPs. They found that the whole-cell catalysts performed better than the commercial enzyme Novozym 435. The presence of water in the crude *Jatropha* oil had significant effects on the rate of methanolysis and whole-cell catalysis performed best in the presence of 5% (v/v) added water. These results suggested that expensive downstream processing steps for potential biodiesel production from *Jatropha* oil can be avoided by using whole-cell biocatalysts for transesterification reaction.

Reports available on transesterification were focused on use of immobilized whole cell biocatalyst which was producing the intercellular lipase. Here in present **Thesis** studies were carried out for the transesterification of non edible oils with the help of whole cell biocatalysts producing extracellular lipase. The first example for the use of a whole-cell biocatalyst for the production of biodiesel came from the results by Ban et. al., in 2001

They have used immobilized mycelium of *R. oryzae* within biomass support particles (BSPs) that have been made out of polyurethane foam. The investigation deals with optimal culture conditions with regard to intracellular lipase production, the effect of pre-treatment methods and the effect of water content on methanolysis. Under the condition of stepwise addition of methanol, the use of BSP-immobilized cells resulted in similar ME content as those reported for extracellular lipases reported by Kaieda, et. al., in 2001.

Among the established whole-cell biocatalyst systems, filamentous fungi have arisen as the most robust whole-cell biocatalyst for industrial applications. **Figure 1.2** shows the comparison between the few steps required for biodiesel production through whole cell processes as compared to the purified lipase.



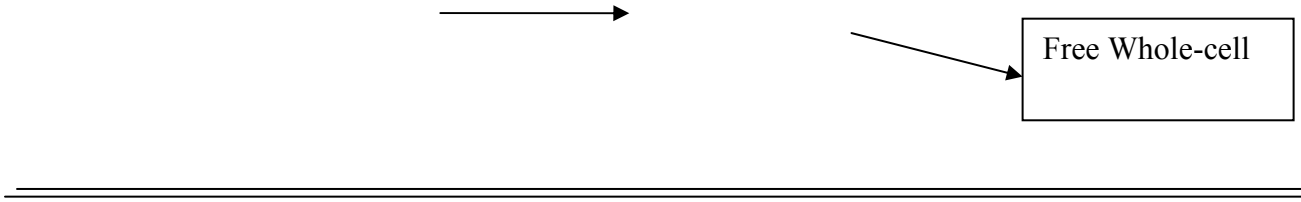


Figure 1.2. Transesterification steps involved in the enzyme and whole cell system.

Comparatively fewer steps are required for lipase producing whole-cell biocatalyst. Whole-cell biocatalyst can also be immobilized to extend their life [Fukuda, et. al., 2008; Hama, et. al., 2006]. If organisms that produce lipases can be readily and cheaply cultured in large quantities, use of such whole-cell biocatalysts offers the promise of inexpensive transesterification of high-FFA wastes, such as yellow and brown grease. Whole-cell enzymes have been used to catalyze a variety of reactions [Yamaji, et. al., 2000; Liu, et. al., 1999 and 2000]. Among the established whole-cell biocatalyst systems, filamentous fungi have arisen as the most robust whole-cell biocatalyst for industrial applications. Table 3 describes various examples of the various whole cell biocatalysts.

Table 1.4. Comparison of biodiesel fuel production methods that used different whole-cell biocatalysts

Whole-cell biocatalyst	**Oil**	**Alcohol**	**Solvent**	**ME (%)**	**Temp.**	**Ref**
BSPs with R. oryzae	Soybean	Methanol	None	90%	32°C	Ban, et al. 2001
BSPs with R. oryzae	Soybean	Methanol	None	90%	35°C	Hama, et al. 2007
BSPs with R. oryzae	Soybean	Methanol	t-butanol	72%	35°C	Wei, et al. 2007
BSPs with R. oryzae	*Jatropha*	Methanol	None	89%	30°C	Tamalampudi, et al 2008

BSPs with R. oryzae	Rapeseed (refined)	Methanol	t-butanol	60%	35°C	Li, et al. 2007
BSPs with R. oryzae	Rapeseed (crude)	Methanol	t-butanol	60%	35°C	Li, et al. 2007
BSPs with R. oryzae	Rapeseed (acidified)	Methanol	t-butanol	70%	35°C	Li, et al. 2007
Mycelium of R. chinensis	Soybean	Methanol	None	86%	NA	Qin, et al. 2008
S. cerevisiae (Intracellular ROL)	Soybean	Methanol	None	71%	37°C	Matsumoto et. al. 2001
S. cerevisiae (cell surface ROL)	Soybean	Methanol	None	78%	37°C	Matsumoto, T. et al. 2002

Matsumoto, et. al., in 2002 developed a yeast cell surface display system for lipase from *R. oryzae* based on the FLO1 gene that encodes a lectin-like cell-wall protein. This gene composed of several domains, including a secretion signal and flocculation functional domain. In the, lipase displaying system, the N-terminus of ROL with a prosequence (ProROL) was fused to the FLO1p flocculation functional domain. The use of yeast cells that display FSProROL and FLProROL led to a ME content of 78.6% after 72 h or 73.5% after a three-step addition of methanol, respectively [Matsumoto et. al., in 2002]. A yeast whole-cell biocatalyst that over produced intracellular lipase from *R. oryzae* produced 71% methyl esters after 165 h of reaction and it is shown in **Figure1.3.** [Matsumoto, et. al., 2001]. Previous reports on *R. oryzae* whole cell biocatalyst showed good conversion than this genetically engineered yeast. Following are the few reports describing wild type *R. oryzae* based transesterification. Li et. al., in 2007 and 2008 reported soybean oil transesterification using *Rhizopus oryzae* IFO 4697 whole cells which yielded 86% methyl esters, similarly *Rhizopus oryzae* cells immobilized within biomass support particles (BSPs) gave 83% conversion as reported by Ban et. al. 2001. Another report of same source of *Rhizopus oryzae* described biodiesel production from

oleic acid in *tert*-butanol medium with 90% methyl ester yield from soybean oil [Wei, et. al., 2006].

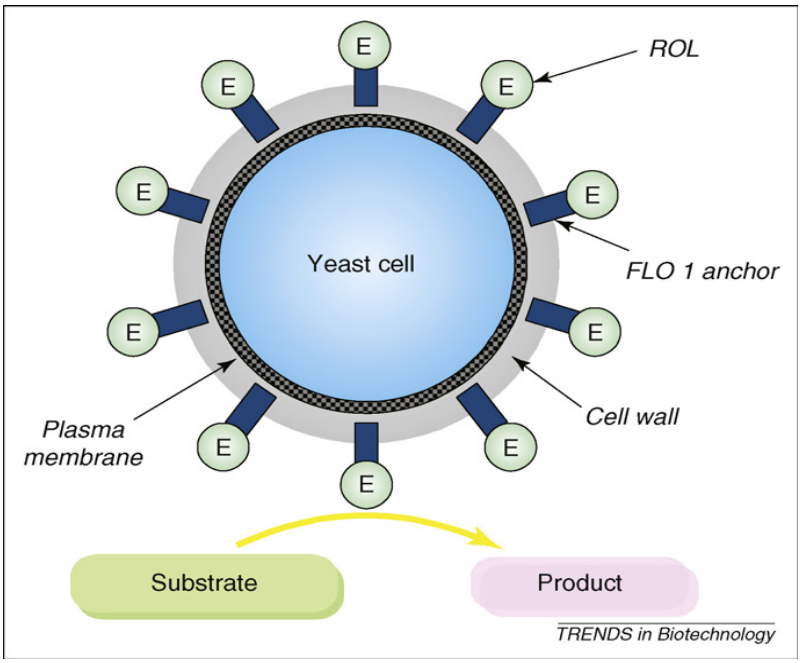


Figure 1.3. Schematic diagram of a yeast whole-cell biocatalyst displaying ROL via an FOL 1 anchor.

1.4. Use of non-edible oils for transesterification:

The use of non-edible vegetable oils when compared with edible oils is very significant in developing countries because of the tremendous demand for edible oils as food, and they are far too expensive to be used as fuel at present [Shah, et. al., 2004]. Considering these drawbacks of vegetable oils non-edible oils such as *Pogamia pinnata* (Karanja or Honge), *Jatropha curcas* (*Jatropha* or Ratanjyote), Argemone, Castor, Sal, etc.and *Madhuca indica* (Mahua) etc., which could be utilized as a source for production of oil [Vasudevan and Briggs, 2008]. However, *Jatropha curcas* and *Pongamia pinnata* (Karanja) has been found most suiTable for the purpose of production of renewable fuel as biodiesel. Tamalampudi et al. in 2008 were the first to utilize whole-cell catalyzed

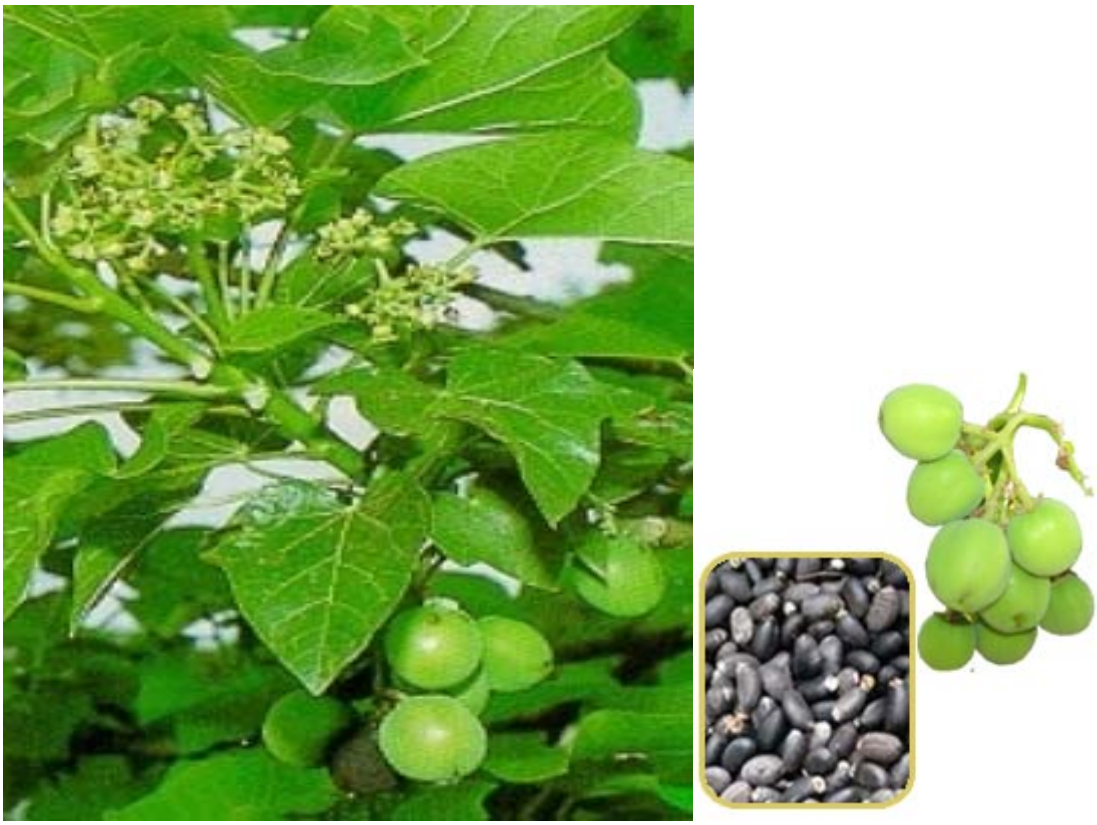
alcoholysis of the non-edible *Jatropha* oil with *R. oryzae* cells that had been immobilized into BSPs. They found that the whole-cell catalysts performed better than the commercial enzyme Novozym 435. The presence of water in the crude *Jatropha* oil had significant effects on the rate of methanolysis and whole-cell catalysis performed best in the presence of 5% (v/v) added water. By contrast, Novozym 435 activity was severely inhibited by the presence of added water and required nearly anhydrous media for efficient catalysis. These results suggest that expensive downstream processing steps for potential biodiesel production from *Jatropha* oil can be avoided with whole-cell biocatalysts.

Present research deals with the studies on transesterification on *Jatropha* and *Ponagmia* oil with the whole cell biocatalyst of newly isolated strain of *Rhizopus.* Non-edible oils such as *Jatropha* (*Jatropha curcas L.)* and *Pongamia* (*Pongamia pinnata*) are getting importance due to its high fatty acid content. This presents these oil plants as a best fit for biodiesel production without altering existing commodity demand-supply equations. Fatty acid composition of the both the oil is as listed below **(Table 1.5).**

Fatty acid	*Pongamia*	*Jatropha*
Oleic	**44.5-71.3**	**37.0-63.0**
Linoleic	10.8	19.0-41.0
Arachidic	2.2-4	7.0-0.3
Palmatic	3.7-7.9	12.0-17.0
Stearic	2.4-8.0	5.0-9.7
Eicosenoic	9.5-12.4	-
Behenic	4.2-5.3	-

Table 1.5. Fatty Acid composition of *Pongamia* and *Jatropha* oil.

Jatropha curcus L. (Euphorbiaceae) is a native of tropical America, but now thrives in many parts of the tropics and sub-tropics in Africa/Asia. **Figure 1.4**. shows the Jatropha plant, fruit and seeds. It is a tropical plant that can be grown in low to high rainfall areas and can be used to reclaim land, as a hedge and/or as a commercial crop. [Openshaw, 2000]. The seeds contain 4–40% viscous oil known as 'curcas oil'. [Datta; et. al., 2007].The oil is high in cetane value and can be used in diesel engines after transesterification to a biodiesel fuel [Openshaw, 2000].



A B

Figure 1.4. A.*Jatropha* curcus plant ; B *Jatropha* fruit and seeds

Pongamia pinnata (L.) Pierre is a fast-growing leguminous tree with the potential for high oil seed production and the added benefit of the ability to grow on marginal land [Scott et. al., 2008]. **Figure 1.5** shows the *Pongamia* pinnata inflorucence and *Pongamia* seeds. The genus is distributed in tropical Asia, Australia, Polynesia, Philippine and Islands. Bhattacharyya and Bhattacharyya, in 1999 is reported that a dry seed of *Pongamia* contains more than 45% of oil. These properties support the suitability of this plant for large-scale vegetable oil production required by a sustainable biodiesel industry [Scott, et. al., 2008].

A B

Figure 1.5. A. *Pongamia pinnata* inflorucence B. *Pongamia* seeds

Conclusion:

In recent years there has been lot of research going on the alternative fuel sources considering the increased demand for fossil fuel and its limited availability. The continuous use of petroleum intensifies problems of air pollutants and carcinogens. There is need to explore other alternative fuel such as biodiesel. Transesterification of oils yield alkyl esters of long chain fatty acids which is a best alternative for fossil fuel. Triglycerides of oil are converted into biodiesel in presence of catalyst and alcohol. Transesterification reaction takes place in presences of alcohol and catalyst, which can be either chemical or biological. Considering various drawbacks of chemical catalysis, biological catalyst is batter alternative. There is increase in demand for whole cells as biological catalyst as compared to enzymes due to laborious purification steps of the enzyme catalysts. Whole cells with high lipase activity can be used as catalysts for the effective and economical biodiesel production. Therefore there is a need for continuous research in this area for new sources of lipase producing organisms. Non-edible oils such as *Jatropha* (*Jatropha curcas L.)* and *Pongamia* (*Pongamia pinnata*) are having high fatty acid content and these oils can be used for biodiesel production. Alcohol required for the reaction is four times more than oil. This alcohol has to be fed intermittently to get complete transesterification which adds in the cost of biodiesel production. Still much work has to carried out in this direction for new lipase sources and also fulfilling the alcohol demand required for the process.

In this context, if alcohol required for transesterification reaction is produced simultaneously using fermentative yeast then the processes can be made cost effective.

Simultaneous alcohol production and transesterification is possible in a single pot in controlled conditions.

Chapters II:

Screening and Optimization of Lipase Producing Fungus and Yeast

Kantak J B, Bagade A V, Mahajan S A, Pawar S P, Shouche Y S and Prabhune A A. (2011) Appl Biochem Biotechnol. 164:969-978.

Abstract:

A lipolytic mesophilic fungus which produces lipase extracellularly was isolated form soil samples of oil spillage areas. The isolate was identified by ITS1-5.8S-ITS4 region sequencing of ribosomal RNA method. Based on Phylogenetic analysis and ITS1-5.8S-ITS4 region sequencing, this microorganism JK1 belongs to genus *Rhizopus* and clades with *Rhizopus oryzae*. A phylogenic tree was constructed based on BLAST analysis using MEGA version 4 software and bootstrap method. Closest phylogenetic relatives of the isolate were identified from the phylogenetic tree.

Culture conditions for maximum lipase production where studied. Optimization of fermentation conditions resulted in enhancement in the production of lipase from this strain. The highest lipase production was observed in mineral medium with corn steep liquor as nitrogen source and glucose as carbon source. Maximum lipase production was observed in 72h, which is about 865U/mL. In the present chapter, screening, isolation, identification and standardization of fermentation conditions for the maximum production of lipase were studied. Optimization of fermentation conditions resulted in 16 fold enhancement in enzyme production.

2.1. Introduction:

Lipases (glycerol ester hydrolyses EC 3.1.1.3) catalyse the hydrolysis of triglycerols in to free fatty acids, glycerol as well as mono and di-acyl glycerols. Another interesting feature of lipase is its ability to synthesize ester bonds in non aqueous medium (Hasan

et. al., 2006; Hiol *et. al.,* 1999; Abbas *et. al.*, 2002; Fukuda *et. al.,* 2001). As described in Chapter I. lipases have various commercial applications in different industries such as food, fine chemicals, detergents, waste water treatment, cosmetics, pharmaceuticals as well as leather processing (Hasan *et. al.* 2006; Abbas *et al.* 2002).

There are various microbial sources of lipase producers among which fungi are preferred when used in industrial applications. Lipase activity has been detected in various species of *Rhizopus* such as *Rhizopus oryzae* (Hiol et. al. 2000; Shukla and Gupta 2007; Ghorbel et. al., 2005), *Rhizopus homothallicus* (Diaz et al. 2006), *Rhizopus oligosporus* **(**Nahas 1988; Iftikhar et. al., 2008**),** *Rhizopus delemar* (Iwai and Tsujisaka 1974), *Rhizopus japonicas* (Aisaka and Terada, 1981*).* Though *Rhizopus* is well reported for the production of lipase very few reports on optimization of fermentation conditions for lipase production are available (Fadiloglu and Erkmen 1999; Hiol et al., 2000; Koblitz and Pastore 2006; Diaz et. al. 2006).

Present Chapter describes screening, isolation and identification of a novel mesophilic fungus *Rhizopus* strain *JK-1* from soil samples of oil spillage areas which produces extracellular lipase constitutively. The effect of different cultural conditions like pH, temperature, time and aeration on lipase production were investigated under shake flask conditions. The effect of carbon and nitrogen sources on enzyme production was also investigated.

2.2. Materials and Methods

2.2.1. Materials

Yeast extract, peptone, and sodium chloride, were obtained from Hi-media, India. Para-nitrophinol palmitate (pNPP) and Bovine serum albumin were obtained from Sigma.

Rhodamine B, 2-propanol and Olive oil were purchased from Merck, India. All other chemicals were analytical grade compounds. All media were prepared in distilled water and buffers in glass distilled water.

Cornsteep liquor (CSL; 50 % dry solids content) was a gift of Hindustan Antibiotics, Pune. CSL was pretreated by suspension of 50 g of liquor in 80 ml of water; the diluted solution was adjusted to pH 7.0 with NaOH, made up to 100 ml; steamed for one h, cooled and clarified by centrifugation (Pundle & SivaRaman, 1994).

2.2.2. Methods

2.2.2.1 Source and Isolation of Lipase producer:

Five different soil samples were collected from places contaminated with oil near lamps in the local temples from Pune, India. The samples were collected in 10 ml MGYP containing sterile *Pongamia* oil (3%). The tubes were incubated at 30°C on a rotary shaker at 180 rpm for 24 h. Further isolation was done on PDA plates.

2.2.2.2. Rapid Screening of lipase producer:

Fifteen different fungal isolates were screened for lipase production on Rhodamine B agar plates. The fungal isolates were inoculated on media of the following composition (g/L) Nutrient broth (Hi Media), 8.0; sodium chloride, 4.0; agar, 10.0. The medium was adjusted to pH 7.0, autoclaved and cooled to 60ºC. Olive oil (31.25 ml) and 10 ml of Rhodamine B solution (0.001%, w/v) were added with vigorous stirring and emulsified by mixing for 1 min. Plates were incubated at 30°C for 48h and irradiated with UV light at 350 nm for orange fluorescent halo corresponding to lypoliytic activity. (Kouker and Jaeger, 1987). Isolate which showed significant zone of florescence indicative of lipase production, was selected for further.

2.2.2.3. Microorganism

Rhizopus strain *JK 1* was maintained on MGYP (Malt Extract- 0.3%, Yeast Extract- 0.3%, Peptone- 0.5% and Glucose-2%) medium slants stored at 4°C and subcultured every 15-30 days. 10 ml sterile medium was inoculated with spores (approximately 10^6/ml) from fresh agar slant culture and incubated for 48 hr at 30°C and 180 rpm. This seed culture was used throughout the studies.

2.2.2.4. Lipase Assay:

The lipase activity was analyzed spectrophotometrically measuring the increment in the absorption at 410 nm promoted by the hydrolysis of pNPP (Winkler and Stuckmann, 1979). The reaction mixture consisted of 0.1 ml of diluted supernatant broth, 0.9 mL of pNPP substrate solution and 1 mL of phosphate buffer (0.05 M, pH 7.0). It was incubated at 30°C for 30 min, followed by addition of 2 ml 2-propanol. The absorbance was measured at 410 nm. The substrate solution containing 30 mg pNPP, 10 mL 2-propanol, 0.1 mL Triton X-100 in 100 mL phosphate buffer (0.05 M, pH 7.0) was prepared freshly. The lipase unit was defined as the amount of enzyme necessary to hydrolyze 1 μmol of pNPP per minute under the described conditions. The protein in supernatant was estimated by Folin–Lowry method (Lowry et al., 1951). Bovine serum albumin (BSA) was used as standard.

2.2.2.4. Lipase production:

Lipase production by *Rhizopus* strain *JK-1* was conducted in 250mL erlenmeyer flasks with 50mL of the basal medium containing Glucose 1%, Na_2NO_3 0.1%, $MgSO_4$ 0.05, KH_2PO_4 0.1%, Peptone 3% inoculated with 10% seed culture prepared in malt extract

0.3%, yeast extract 0.3% ,peptone 0.5% and glucose 2%. Flasks were incubated at 30°C on rotary shaker with 180rpm for six days. Enzyme production was monitored after every 24h. Centrifuged culture broth was used as enzyme source. Mycelial dry weight was determined by filtering the culture medium and drying the mycelia at 80ºC till constant weight was obtained.

2.2.2.5. Identification:

DNA isolation:

One gram of cell were lyophilized and the dry mass was homogenized in extraction buffer (containing10 mM Tris, pH 8.0, 100mM NaCl, 1 mM EDTA, 1 % SDS, 50 mM DTT, 1 % β-mercaptoethanol) after mixing equal amount of phenol chloroform (1:1) was added and vortexes gently. After centrifugation for 10min supernatant was removed and above step was repeated again. After centrifugation to the supernatant two volumes of chilled ethanol was added slowly and kept at -20ºC for 20 min. This solution was then centrifuged at 12000rpm for 10 minutes at 4 ºC and the pellet was resuspended in 400µl of TE pH 8 with RNase (20µg/ml), this mixture was incubated at 37 ºC for 30 min. Again precipitation with phenol: chloroform was carried out and centrifuged at 12000 rpm for 10 min. was done at 4 ºC. The upper aqueous layer was transferred to the fresh tube in to it 3M sodium acetate and 1 ml ethanol was added and mixed by inversion. This was incubated at room temperature for 5 min and centrifuged at 12000 rpm for 10 min. at 4ºC. The Pellet was air dried and dissolved it 200µl of TE pH 8.And quantitation was done by using nanodrop.

DNA amplification and Sequencing:

DNA was isolated by using the method by Sambrook J and Russel DW 2001. PCR amplification conditions using primers for ITS region were 35 PCR cycles of 94°C 1min, 55°C 1min and 72°C 1min (Baldwin, B.G. 1992). Amplified DNA was checked on 1% agarose gel and purified using the PEG-NaCl method. The purified PCR product was rechecked on 1% agarose gel and used for sequencing using ABI Bigdye chemistry version 3.1. Sequences acquired were quality checked manually. CromasPro version 1.34 was used for contig formation and quality trimming. The contig obtained was deposited in NCBI Genbank database available under the accession no HQ222811. Sequences homologous to *JK-1* were obtained using NCBI BLAST. Sequences with high query coverage and homology were selected for phylogenetic analysis (Saitou, N & Nei, M.,1987). Multiple sequence alignment was done using ClustalX (Larkin et. al., 2007), the aligned sequences were trimmed using DAMBE and the phylogenetic tree was constructed using MEGA version 4 (Tamura et. al., 2007).

2.2.2.6. Optimization of Fermentation Parameters:

Optimization of different nutrients and physical parameters for lipase production were studied by maintaining all factors constant except the one being studied. Fermentation was carried out in 250mL erlenmeyer flasks containing 50 ml of sterilized basal media inoculated with 10% inoculum. Flasks were kept on shaker at 180 rpm, 30 °C for

144 h.

2.2.2.7. Effect of initial pH

Production of lipase at different pH values was determined by cultivating the isolate in different initial pH values, in the range pH 5.5 to 8.5 of the basal medium. Cells were

grown in 50 ml of the selected medium for six days on a rotary shaker at 180 rpm. Culture broth was used as the source of extracellular enzyme for determination of lipase activity at different pH values.

2.2.2.8. Effect of incubation temperature

The influence of temperature was studied by cultivating the isolate at various incubation temperatures ranging from 25°C to 45°C. Cells were grown in 50 ml of the selected medium for six days (180 rpm) at the above mentioned temperature range. Lipase activity was determined in the culture broth after each 24h with the help of assay described earlier.

2.2.2.9. Time kinetics profile

Cells were grown in basal medium at 30°C on a rotary shaker at 180 rpm. Two microliters of sample was withdrawn at intervals of 24 h and lipase activity was determined. Lipase production profile and cell growth of the culture were monitored under shake flask conditions up to 6 days.

2.2.2.10. Effect of carbon source

The effect of different carbon sources on production of lipase activity in shake flasks were assayed by substituting the carbohydrate from basal medium with various carbon sources (1.0 %). Simple carbon sources like glucose, sucrose, maltose and glycerol and complete lipogenic source olive oil were used. Lipase production was monitored under shake flask conditions with each carbon source up to 6 days. Minimal medium without carbon source was served as control.

2.2.2.11. Effect of Nitrogen source

The influence of various nitrogen sources on cell growth and enzyme yield were also tested. Effect of different nitrogen sources such as peptone, tryptone, casamino acids, yeast extract and corn steep liquor were studied on lipase production. Cells were grown in 50 ml of the selected medium with different nitrogen source for six days at 180 rpm). Concentration of nitrogen source was used 3%. Lipase production in culture broth was determined as described earlier. Minimal medium without nitrogen source was served as control.

3.2.2.12. Effect of dispensing volume

Erlenmeyer flasks (250 ml), containing different volume (25-125 ml) of medium were used to standardize effect of aeration for lipase production. 10 % (v/v) inoculum size was used to inoculate flasks containing various volumes of medium in the range of 25-125ml. The inoculated flasks were kept for incubation at 30°C on a rotary shaker at 180 rpm for 6 days. Lipase activity was determined by the assay described earlier.

2.3. Results and discussion:

2.3.1. Source, screening and Isolation of Lipase producer:

Five samples were collected from different oil contaminated soil near lamps in the local temples from Pune, India and enriched in MGYP supplemented with *Pongamia* oil. Among these samples 15 fungal isolates were screened for lipase production on Rhodamine B agar plates. The best lipase producer named as JK-1 with significant fluorescence halo (**Figure 2.1**) was selected for further studies. Microscopic observations isolate were presented in **Figure 2.2.**

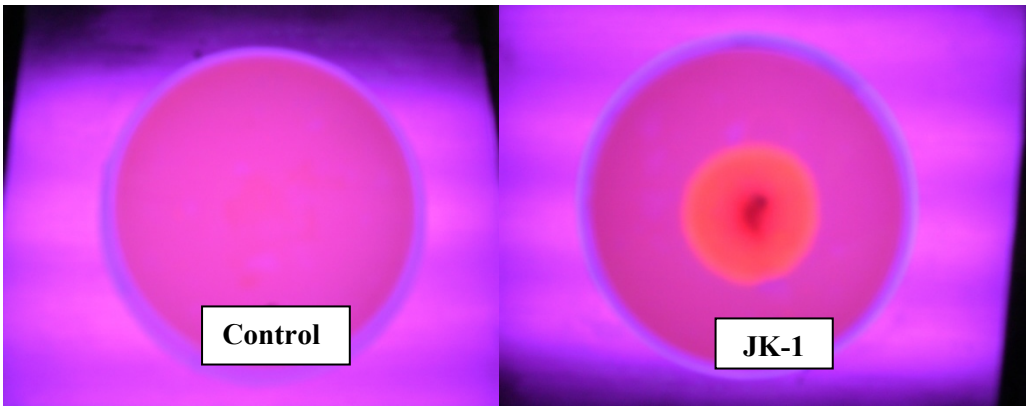


Figure 2.1: Plate assay: Zone of fluorescence around the colony of test organism against the Rhodamine B agar indicated the lipase production when compared with control.

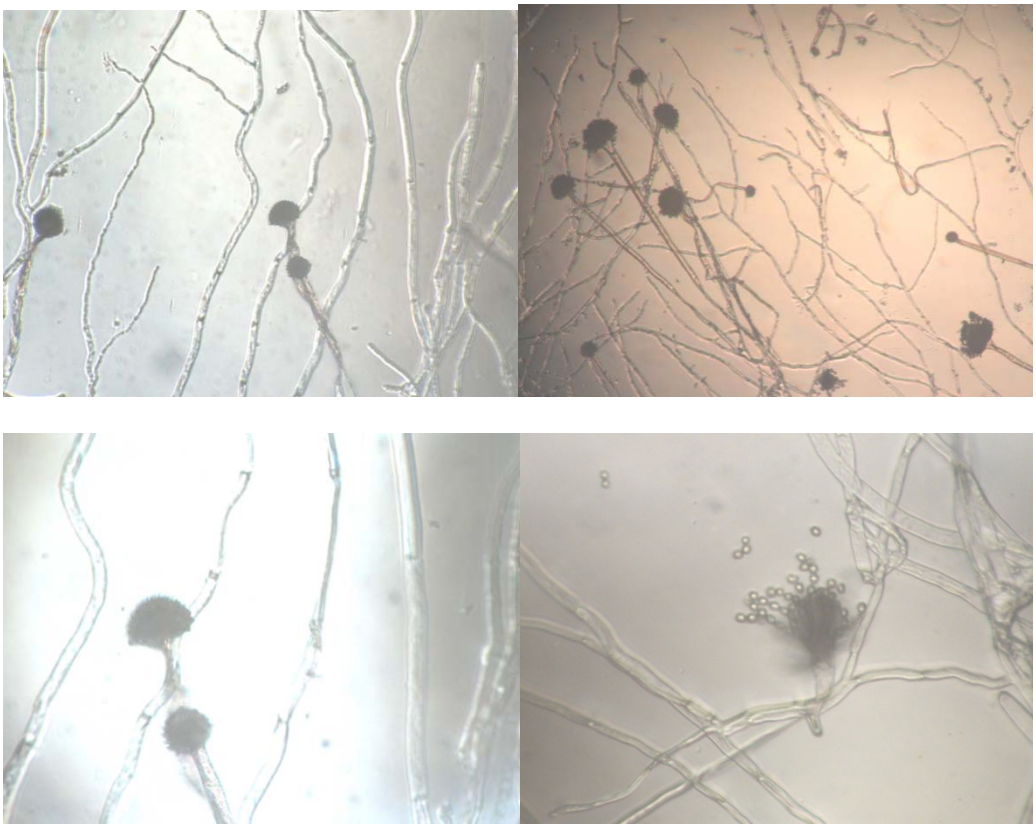


Figure 2.2. Optical microscopic (Zeiss; Carl Zeiss India Pvt. Ltd.) images of isolate JK 1. Magnifications of the images are 40 X.

2.3.2. Identification of Lipase producer:

The phylogenetic analysis of strain JK-1 ’s ITS sequences, a complete stretch of 553 base pairs, revealed that strain JK-1 clades with *Rhizopus oryzae*. The sequence of JK1’s ITS domain is available in NCBI database under the GenBank accession number HQ222811.

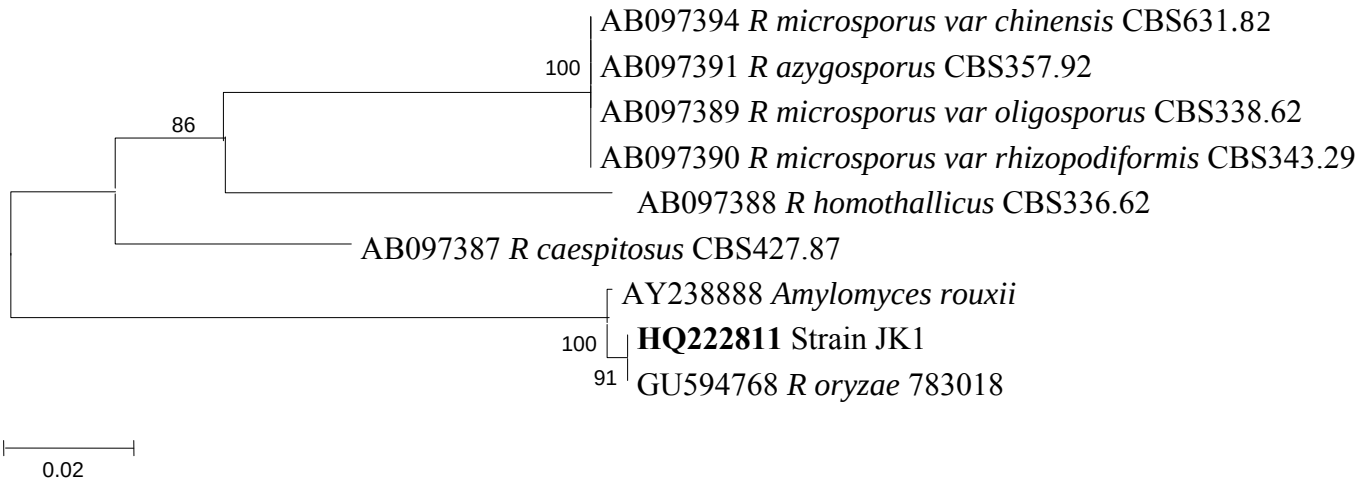


Figure. 2.3. Neighbour-joining phylogenetic tree constructed based on ITS sequences, showing the phylogenetic relationship between Strain *JK-1* and other *Rhizopus* sp. Bootstrap values (expressed as percentages of 1000 replications) >50% are given at nodes. The bar represents 2 substitutions per 100 nucleotides.

2.3.3. Optimization of Fermentation Parameters

The production of high titres of any enzyme by optimizing the growth parameters is of prime importance in enzymology. The optimization of various nutritional and physical parameters to which an organism exposed is known to significantly increase the product yield. To achieve the maximum production of enzyme from a novel source, culture

conditions were optimized. Optimum lipase production was observed in basal medium containing Glucose 1%, Na_2NO_3 0.1%, $MgSO_4$ 0.05, KH2PO_4 0.1%, peptone 3%. This medium was used for the rest of the experiments. Strain *JK-1* was assigned to genus *Rhizopus* based on the phylogenetic analysis of ITS1-5.8S-ITS4 region sequence, a complete stretch of 553 base pairs (Abe *et. al.,* 2006). Isolate *Rhizopus* strain *JK-1* produces this enzyme extracellularly with 862U/mL (Specific activity 30.38 units) under optimum conditions. The property of *Rhizopus* strain *JK1* to produce extracellular lipase is having its own significance over reported intercellular lipases by minimizing the downstream processing of enzyme (Essamri *et. al.,* 1998). This is second highest lipase activity report when compared with species so far reported (Essamri *et. al.*, 1998; Hiol, *et. al.*,2000; Shukla and Gupta, 2007; Ghorbel et. al., 2005; Diaz et. al., 2006; Nahas, 1988; Iftikhar *et. al.*, 2008**;** Iwai and Tsujisaka 1974; Aisaka and Terada, 1981**.** There are reports on lipase production by *Rhizopus sp*. but the enzyme unit activity reported is very low under optimum fermentation conditions (Shukla and Gupta, 2007; Hiol *et. al.,* 1999; Ghorbel *et. al.*, 2005*;* Iftikhar *et. al.,* 2008; Diaz *et. al.,* 2006). Microbial lipase fermentations are affected by the medium pH, temperature, media composition and aeration. Various nutritional and physical parameters were known to be involved in increasing the enzyme activity and it is already known that the culture environment has a dramatic influence on enzyme production (Elibol & Ozer, 2001). Enzyme yields can be enhanced by several fold by providing the suiTable cultural conditions. Since lipase is an industrially important enzyme its production needs to be increased by optimizing the various parameters.

2.3.4. Effect of initial pH

In fermentation its well known that initial pH of the medium strongly influence production of enzymes. The pH affects the ionization and therefore the binding and interaction of enzyme and this includes very basic things such as nutrient availability and solubility. The pH profile for lipase from *Rhizopus* strain *JK-1* was determined in basal medium adjusted to different pH values ranging from 5.5 to 8.5 **(Figure2.4).** Optimum Lipase activity was detected in pH 7.5. Initial pH is very important factor which monitors the extracellular enzyme production. In case of fungi, majority of researchers have reported an acidic pH to be the most appropriate for enzyme production (Hiol et. al., 1999). However Diaz et. al., 2006 and Nahas, 1988 found that optimum pH was 6.5 for lipase production through *R. homothallicus* and *R. origosporus*. But here we are reporting slightly near and above neutral pH 7.5 for optimum lipase production.

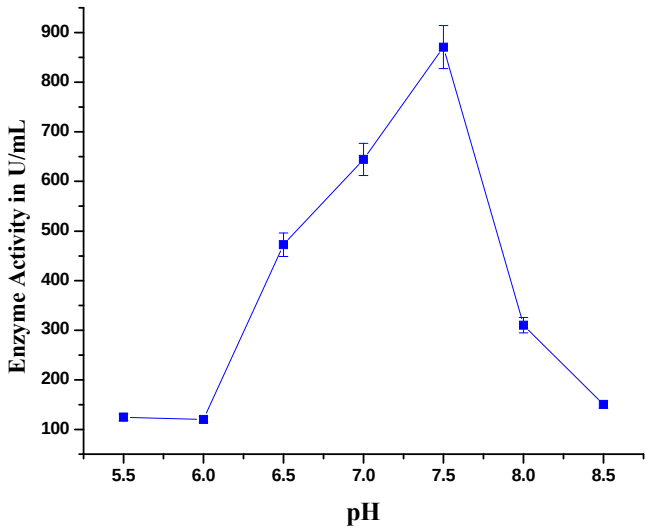


Figure2.4. Effect of initial pH of the culture medium on lipase production by strain *JK-1*

2.3.5. Effect of incubation temperature

The new *Rhizopus* strain was isolated from mesophilic origin so its necessary to check the temperature profile of lipase production of new isolate. Temperature profile for lipase activity showed that enzyme activity was maximum at 30°C when incubated at different temperatures ranging from 25 to 45°C **(Figure2.5).** The culture failed to grow at 25°C and above 45 °C. Though the culture grew at higher temperatures, maximum enzyme production was obtained at 30°C similar results have been found out by Hiol et. al. (1999).

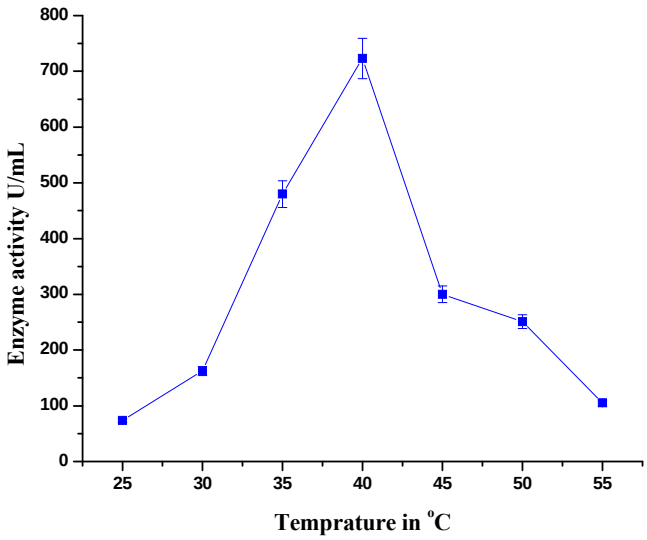


Figure2.5. Effect of incubation temperature on lipase production by strain *JK-1*

2.3.6. Time kinetics profile

Time course for growth of *Rhizopus strain JK 1* and lipase production was observed in shake flask conditions. As shown in **Figure2.6**, the production of lipase increased steadily with the cultivation time and the best enzyme production, about 802U/ml was reached at 7.1 g/l mycelial dry weight after 3 days of cultivation. After three days gradual decrease in enzyme activity was seen. Increase in biomass was seen up to 4 days then culture entered stationary phase and simultaneously enzyme activity was dropped.

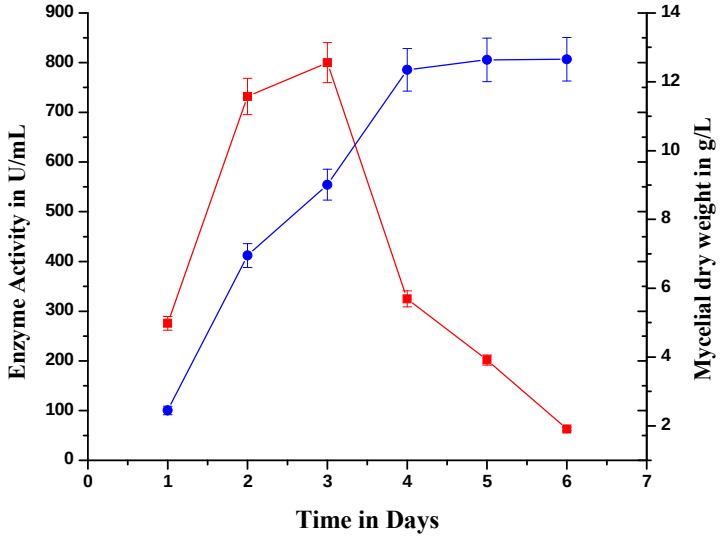


Figure 2.6. Effect of fermentation time on lipase and biomass production at 30 °C, pH 7.5, 180 rpm in basal medium with CSL. Samples were removed after every 24h time intervals and assayed for growth (●) and lipase activity (□) simultaneously

2.3.3.2. Effect of dispensing volume

In any aerobic fermentation airetion is an importent parameter which is to be optimized for maximum production of enzyme. Effect of aeration was studied by dispensing 25-125ml of basal medium in 250ml Erlenmeyer flasks. Maximum lipase production was observed in the flasks containing 50ml basal medium when incubated at 30°C, pH 7.5, at180rpm **(Figure2.7).** The effect of aeration was important for optimum metabolic rates (growth and enzyme production) of microorganism. For aerobic fermentation, oxygen transfer is a key variable and is a function of aeration and agitation. Medium volume plays important role in the mass transfer during fermentation. Low oxygen supplies can alter fungal metabolism and, consequently, the production of lipases (M. Elibol et.al). These facts were true in the lipase production of *Rhizopus* strain JK1 here, we obtained maximum enzyme (862U/ml) in the flasks containing 50ml of basal medium when studied the range of 25-125ml of medium.

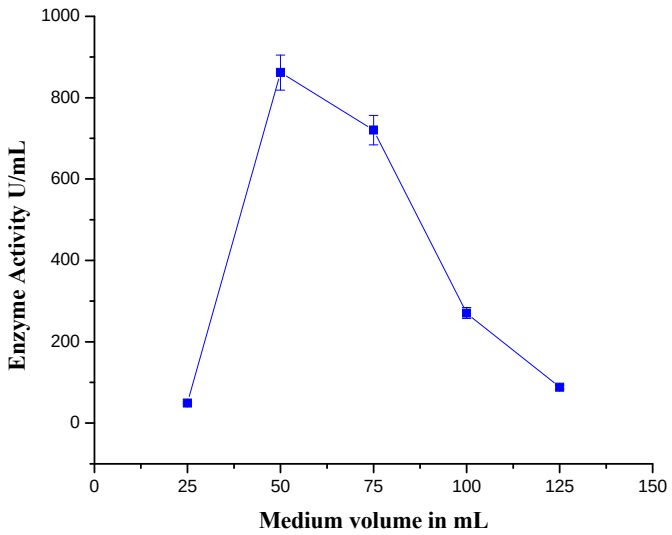


Figure2.7. Effect of aeration on lipase production by strain JK-1

2.3.6. Effect of Carbon source

Carbon source is very important for every fermentation reaction because this was used for the survival of the cells producing different secondary metabolites such as enzymes. Influence of various carbon sources were tested for the production of lipase by *Rhizopus strain JK 1* and the results are depicted in **Figure 2.8.** Glucose was observed as best carbon source among the tested simple and complex carbon sources. However there was increased biomass production in olive oil containing basal medium (data not showed) but no considerable increase in enzyme activity. *Rhizopus* strain JK1 produces lipase constitutively. One percent glucose in the medium shows maximum lipase production. In industrial scale fermentation, inducer's use may cause inconsistencies in medium homogeneity, oil-water surface problems and may involve expensive purification process (Sharma et.al 2001). Therefore, an efficient microbial system that can utilize soluble sugars as carbon and energy sources is quite attractive.

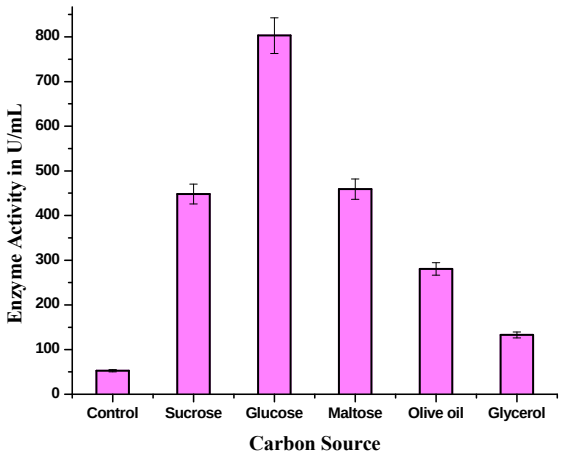


Figure2.8. Effect of carbon source on lipase production during cultivation of strain *JK-1*

2.3.7. Effect of Nitrogen source

Nitrogen source has its own significance on lipase production. Corn steep liquor (3g%) was found to be the best among the nitrogen source tested which gave the best enzyme units up to 870U/mL **(Figure2.9 and 2.10)** with specific activity in the range of 28-31 throughout the study. A sixteen fold increase in lipase production was archived in basal medium with addition of corn steep liquor (3g%) and 1% glucose, pH 7.5 at 30°C in shake flasks condition at 180 rpm after 72 h of incubation. *Rhizopus* strain *JK-1* produces lipase constitutively which eliminates the problem caused by the inducers used in the medium (Sharma *et.al* 2001). Among the various nitrogen sources tested CSL at concentration of 3g% was the best for optimum enzyme production. The nitrogen source is usually the most expensive component of the medium when organic nitrogen sources such as peptone, tryptone, yeast extract, etc are considered. Various *Rhizopus* sp. have been reported using Corn Steep Liquor as nitrogen source but in very high concentration up to 6-7g% (Essamri *et. al.,* 1998). Cost of lipase production can be reduce considerably by the use of inexpensive raw materials such as CSL (at 3 g% concentration), prompting new industrial applications.

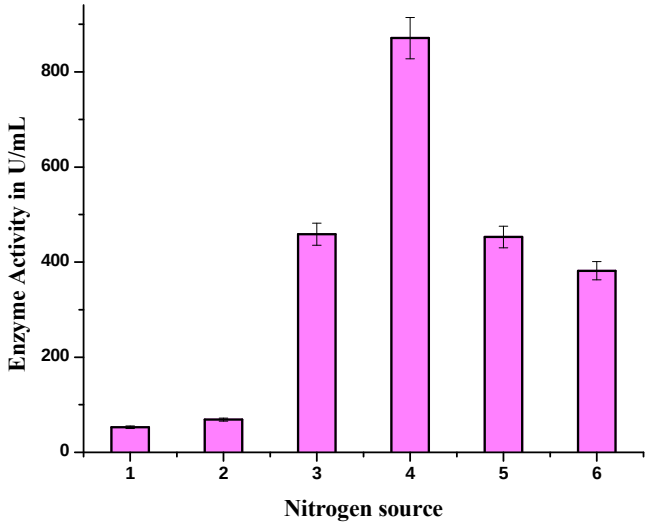


Figure2.9. Effect of nitrogen source on lipase production during cultivation of strain *JK-1***.** The following different nitrogen sources were used 1 Control, 2 Cassamino acids, 3 Peptone, 4 Corn steep Liquor, 5 Tryptone and 6 Yeast extract.

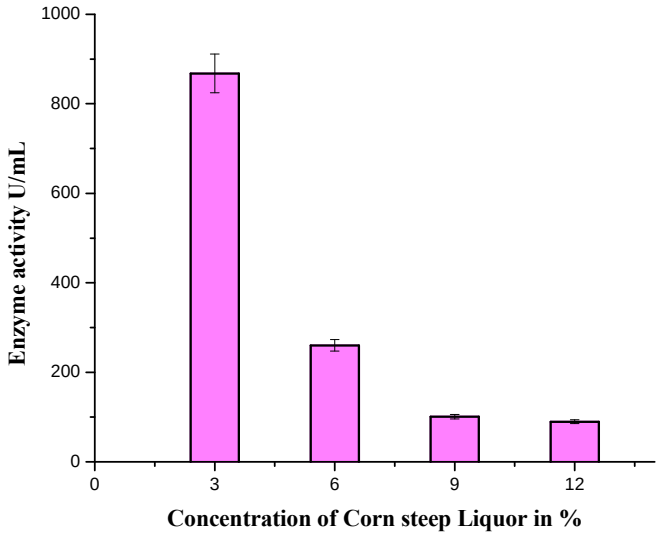


Figure2.10 Effect of CSL concentration on enzyme production

2.4. Conclusion:

In the conclusion, we report a mesophilic *Rhizopus* JK-1 which constitutively produces extracellular lipase. Maximum lipase production was seen when basal medium was supplemented with 1% glucose and 3% CSL with optimum pH, temperature, aeration and incubation time. We are reporting highest lipase units in these optimum conditions prompting cost effective industrial production of this enzyme.

Chapters III:

Production, Purification and Biochemical Characterization of Lipase from new isolate Strain of *Rhizopus*

Kantak J. B. & Prabhune A. A. (2012) Appl Biochem Biotechnol 166:1769–1780

Abstract

An extracellular lipase producing fungus was isolated from oil rich soil. This fungus belongs to genus *Rhizopus* and clades with *Rhizopus oryzae*. Lipase was purified to homogeneity from this novel fungal source using ammonium sulphate precipitation followed by Q-sepharose chromatography. The extracellular lipase was purified 8.6 fold and its enzymatic properties were studied. The molecular mass of the purified enzyme was estimated to be 17 kD by SDS-PAGE and 16.25 kD by MALDI-TOF analysis. The native molecular mass was estimated to be 17.5 kD by gel filtration, indicating the protein to be monomer. Analysis by using SDS-PAGE, MALDI –TOF MS and Gel filtration indicated that the lipase produced by this *Rhizopus* strain JK-1 has the lowest molecular weight lipase of fungal origin reported till today. This is the first report on purification of lowest molecular weight lipase from fungal origin. The optimum pH and temperature for the enzyme catalysis were 7.0 and 40 °C respectively. Enzyme was sTable in pH range 6.0-7.0 and retains 95-100 % activity when incubated at 50 °C for 1 h. The pI of the purified lipase was 4.2. Enzyme was sTable in the organic solvents (30%) such as ethanol, hexane and methanol for 2h.

3.1. Introduction:

Lipases (triacylglycerol ester hydrolases EC 3.1.1.3) represent a group of enzyme having the ability to hydrolyze triglycerides at lipid- water interface. Among the different biocatalysts known, lipases have their own importance for biotechnological applications. Lipase being industrially important enzyme has various applications for synthesis of industrially important products. Lipases show remarkable levels of activity and stability in non-aqueous environments, which in turn is very useful for catalyzing important reactions such as esterification and transesterification [Fukuda, et. al., 2001]. As

described in **Chapter I**, lipases are versatile biocatalysts in the pharmaceutical, food, biofuel, cosmetic, detergent, leather, textile and paper industries [Jaeger and Reetz, 1998; Jaeger, & Eggert, 2002; Fariha, et. al.,2006]. The industrial demand for exploring the highly active preparations of lipolytic enzymes is continuously stimulating the research on novel enzyme sources.

As compared with the different sources of lipases, microbial lipases are currently receiving more attention in the developing field of enzyme technology. There are various microbial sources of lipase producers among which fungi are preferred when used in industrial applications [Essamri, et. al. 1998]. Various *Rhizopus* species were reported for both intracellular and extracellular lipase production [Kantak, et. al., 2011]. Enzymes produced extracellularly have their own benefits, which facilitates easier separation of enzyme from fermentation broth minimizing prolonged purification steps as compared to intercellular enzymes [Rapp and Backhaus, 1992]. As described in **Chapter II**, screening for extracellular lipase producing fungus in our laboratory led to the identification of mesophilic fungus isolated from soil samples of oil spillage areas in Pune, Maharashtra, India. This fungus was identified by ITS1-5.8S-ITS4 region sequencing analysis, and it belongs to genus *Rhizopus* and clades with *Rhizopus oryzae* [Kantak, et. al., 2011]. These studies include purification of an extracellular lipase from newly isolated *Rhizopus* strain JK-1 (HQ222811) to homogeneity and its biochemical and molecular properties in comparison to those of lipases from other fungi of the *Rhizopus* genus.

In the present **Chapter**, studies on purification of the extracellular lipase from new *Rhizopus* strain JK 1 and its biochemical characterization such as molecular weight,

effect of different metal ions and amino acid modifying reagents and effect of organic solvents were described.

3.2. Materials and methods:

3.2.1. Materials

Q-sepharose, p-Nitrophenyl palmitate, bovine serum album (BSA), native and gel filtration molecular weight marker were purchased from Sigma Aldrich and SDS molecular weight markers and pI standards were from Bio-Rad (USA). All other chemicals used were of analytical grade. Corn steep liquor (CSL) was gift from Hindustan Antibiotics Ltd. Pune, India.

3.2.2. Methods

3.2.2.1. Microorganisms

Rhizopus strain *JK 1* was maintained on MGYP (Malt Extract- 0.3%, Yeast Extract- 0.3%, Peptone- 0.5% and Glucose-2%) medium slants stored at 4°C and subcultured every 15-30 days. 10 ml sterile medium was inoculated with spores (approximately 10^6/ml) from fresh agar slant culture and incubated for 48 hr at 30°C and 180 rpm. This seed culture was used throughout the studies.

3.2.2.2. Culture conditions and enzyme production

Rhizopus JK-1 was grown under optimum culture conditions for production of extracellular lipase as described earlier [Kantak, et. al., 2011]. The organism was cultivated in 250 ml Erlenmeyer flasks containing 50 ml of basal medium Containing (g/L) Glucose 10, Na_2NO_3 0.1, $MgSO_4$ 0.05, KH_2PO_4 0.1, CSL 30ml seeded with 48 h grown 10 % inoculum prepared in MGYP (Malt Extract- 0.3 %, Yeast Extract- 0.3 %,

Peptone- 0.5 % and Glucose- 2 %). Flasks were incubated in a rotary incubator for 72 h at 30 °C and 180 rpm.

3.2.2.3. Purification of lipase

All purification steps were carried out at 4 °C except when stated otherwise. At the end of the cultivation period of six days, mycelia were removed by filtration (Whatman paper 1). The filtrate was centrifuged at 10,000 rpm for 20 m to remove the suspended mycelia and the resulting supernatant was lyophilized (Christ, USA) for 5 h to concentrate the enzyme in broth, this was used as starting material for lipase purification. The cell - free extract was subjected to ammonium sulfate fractionation and the fraction obtained between 30 – 90 % saturation was collected, dissolved in the minimum volume of 50 mM phosphate buffer, pH 7.0, and dialyzed overnight against 100 volumes of 10 mM phosphate buffer , pH 7.0. The dialysate was clarified by centrifugation and then subjected to ion exchange chromatography.

Ion exchange chromatography

The crude enzyme was subjected to anion exchange chromatography using Q- Sepharose column (2 x 6 cm) which was pre-equilibrated with 50 mM phosphate buffer pH 7.0. The column was washed with the same buffer to remove unbound protein. Flow rate was maintained 12 ml /h. Active fractions were pooled and concentrated on Speed Vac (LABCONCO) and rechromatographed on freshly packed Q- Sepharose column of same dimensions as described previously. Fractions having high specific activity were collected and stored in aliquots at -20 °C to avoid loss of activity due to freezing & thawing till required.

3.2.2.4. Lipase activity measurement and protein content

The activities of enzyme were analyzed spectrophotometrically measuring the increment in the absorption at 410 nm promoted by the hydrolysis of pNPP [Winkler & Stuckmann, 1979]. Assay mixture contains 0.1 ml of diluted enzyme sample, 0.9 ml of pNPP substrate solution and 1 ml of phosphate buffer (50 mM, pH 7.0) incubated at 30 °C for 30 m, followed by addition of 2 ml 2-propanol. The absorbance was measured at 410 nm. The substrate solution containing 30 mg pNPP, 10 ml 2-propanol, 0.1 ml Triton X-100 in 100 ml phosphate buffer (50 mM, pH 7.0) which was always freshly prepared. One lipase unit was defined as the amount of enzyme necessary to hydrolyze 1 μmol of pNPP per minute under the specified conditions. The protein was estimated by Folin–Lowry method [Lowry,et. al., 1951], Bovine serum album (BSA) was used as a standard. In chromatography experiments, the protein content of each fraction was routinely estimated.

3.2.2.5. Analytical gel electrophoresis and Isoelectric Focusing

SDS-PAGE :

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE, 10 %) was carried out by the method described by Laemmli UK in 1970, to check the purity at each step of purification and to determine the molecular mass of the purified enzyme. Silver staining was used to visualize protein bands on the gels [Morrissey, 1981]. Molecular weight was estimated based on concurrently electrophoresed marker proteins (Bio-Rad). The Molecular weight markers used for the SDS-PAGE were phosphorylase b (97.4 kDa), bovine serum album (66.2 kDa), ovalbum (45.0 kDa), carbonic anhydrase (31.0 kDa) and Lysozyme (14.4 kDa). The precise molecular weight of protein was determined using a MALDI-TOF mass spectrometer (Applied Biosciences).

Isoelectric Focusing:

Isoelectric focusing using polyacrylamide gel (10 % w/v) was performed according to the method of Vesterberg described in 1972. The electrophoresis was carried out in duplicate tube gels using ampholines of pH range 3-10. For IEF, 200 µg of purified protein was used. A constant current of 15 mA and an initial voltage of 200 V for 3 h, and then 300 V were applied for 16-18 h at 10 °C. The tube gels were removed at the end of run and were cut into small segments which were subsequently put into tubes for elution with glass distilled water. Elutes of the gel slices were checked for pH and enzyme activity.

3.2.2.6. Determination of molecular mass by gel filtration:

The molecular weight of native protein was calculated according to Andrews in 1964. Gel filtration was carried out using Sephadex G-200 column (100 x 1 cm), at a flow rate of 0.1 ml m^{-1} with 50 mM phosphate buffer at pH 7.0. The relative molecular weight (Mr) was determined by using different protein standards; β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum album (66 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa).

3.3.2.7. Matrix-assisted laser desorption ionization / time-of-flight mass spectrometry (MALDI-TOF MS)

Molecular mass of the purified lipase was also determined by matrix-assisted laser desorption ionization / time-of-flight (MALDI-TOF) on a Voyager DE-STR (Applied Biosystems) system equipped with a 337-nm nitrogen laser. Spectra were acquired in the range of 10 to 40 kDa. The analysis was performed in four replications. The instrument was calibrated with myoglobulin and bovine serum album. Five microliters of protein

was mixed with 35 µl of freshly prepared sinapinic acid (15 mg/ml in 30 % acetonitrile) and loaded on to the stainless steel MALDI plate and dried for 10 m at 37 ºC.

3.2.2.8. Effects of temperature and pH on lipase activity and stability

For determination of the optimum temperature, 200 µg of the enzyme was assayed over different temperatures in the range of 25-55 °C at pH 7.0 in 50 mM phosphate buffer. To ascertain the thermostability, the enzyme was incubated at 30-60 °C from 1-5 h in 50 mM phosphate buffer pH 7.0 and relative activity was estimated under standard assay condition as described earlier.

To study the optimum pH, 200 µg of enzyme was incubated at 30 °C with 50 mM buffer of pH ranging from 4.0-10.0. The following buffers were used, acetate buffer (pH 4.0–6.0), phosphate buffer (pH 6.0–8.0), Tris-HCl (pH 8.0–9.0) and carbonate–bicarbonate buffer (pH 9.0–10.0). For pH stability, 200 µg enzyme was incubated in 50 mM buffers as mentioned above in the range of pH 4.0-10, for 1 h at 30 °C. Residual activity was measured by standard enzyme assay as described earlier. All the experiments were done in triplicates.

3.2.2.9. Effect of various metal ions and amino acid modifying reagents on lipase activity

The effect of different metal ions on lipase activity was assessed. Two hundred microgram enzyme was incubated with various metal ions with effective concentration of 10 mM for 1 h at 30 °C and the residual enzyme activity was determined by standard assay method. Resulting enzyme activities were compared to that of the standard enzyme reaction carried out in controlled conditions without any metal salt.

Various potential amino acid modifying reagents such as *N*-bromosuccinamide, phenylymethylsulphonyl fluoride, 5, 5′- dithiobis- (2-nitrobenzoic acid), phenyl glyoxal, N-Acetylinidazole, Woodwards reagent K, citraconic anhydride, trinitrobenzene sulphonic acid were tested for their effect on lipase activity. For this studies 200 µg of homogenous preparation of the enzyme was incubated with various concentrations of above mentioned reagents at 30 °C for 30 m and residual lipase activity was measured. The percent residual enzyme activity was determined by standard enzyme assay with reference to the activity of the enzyme in a reaction without addition of any modifying reagent. All the experiments were carried out in triplicates.

3.2.2.10. Stability of lipase in different organic solvents

Lipase is industrially important enzyme and some of the applications like chiral resolution, textile industries, detergents and transesterification require organic solvent tolerant lipases. It is very important to understand the stability of a novel lipase in different organic solvents. The enzyme was incubated in the presence of various organic solvents to study its stability. Two hundred µg of enzyme was mixed with different concentration of respective organic solvents from 10- 30 % v/v and incubated at room temperature for 1-2 h. Residual enzyme activity was determined by standard enzyme assay with reference to the activity of the enzyme in a reaction without the addition of any organic solvent under identical conditions.

3.2.2.11. Statistical analysis:

All the experiments were carried out in triplicates and values are mean ± standard deviation (SD). SD was calculated by using formula $\sigma = \sqrt{\sum (x - \bar{x})^2 / N}$. Where σ

is the standard deviation, x is each value in the population, x is mean and N is number of observations.

3.2.2.12. Enzyme-Catalyzed Transesterification of Oleic Acid

Oleic acid (1 g) and ethanol were taken in the ratio of 1:4 in a 25-ml stopper bottle and incubated under shaking condition (160 rpm) at 30 °C. After 30 min incubation, purified lipase (900 U) was added to catalyse the transesterification reaction. The progress of the reaction was monitored by removing aliquots (100 μL) at various time intervals up to 48 h. The upper layer was centrifuged and diluted in n-hexane and used for the ethyl ester analysis by gas chromatograph with flame ionization detector (Master GC DANI, Italy). The capillary column (HP-INNOWax, Agilent Technology, USA) used had a length of 30 min. with an internal diameter of 0.25 mm. Nitrogen was used as the carrier gas at a constant flow rate of 4 kg cm−2. The column oven temperature was programmed from 80 °C to 210 °C (at the rate of 50 °C/m) and held at this temperature for 6 min. The oven temperature is further increased to 230 °C (at the rate of 5 °C/m) with the same temperature held for 8 min. The injector and detector temperatures were 230°C and 240 °C, respectively.

3.3. Results and discussion:

3.3.1. Purification:

A summary of purification of extracellular lipase from novel *Rhizopus* strain *JK-1* is presented in **Table 3.1.** Protein was purified to homogeneity using ion exchange chromatography on Q-Sepharose column. Lipase from *Rhizopus* strain JK- 1 showed very weak binding with anionic matrix Q-Sepharose, which was evident that enzyme which got eluted with plain 50 mM phosphate buffer without any gradient. The pooled active fractions were concentrated and further rechromatographed on another freshly

packed and pre-equilibrated Q-sepharose column. As a result of purification using this procedure 23.37 % yield was obtained and the protein was purified almost 9 fold (8.57). Present lipase get purified with very simple steps as compared to the multistep purification of lipase from other *Rhizopus* species [Hiol, et. al., 2000; Iwai and Tsujisaka 1974; Diaz , et. al., 2006; Sun, et. al., 2009]. The purified lipase moved as a single band in native PAGE indicating its homogeneity. As shown in Figure 1, the purified enzyme appeared as a single band in SDS-PAGE with molecular mass around 17 kDa. Upon gel filtration **(Figure 3.2)**, a single peak of lipase activity was eluted and corresponded to a protein of molecular mass around 17.5 kDa. MALDI-TOF MS analysis revealed that the purified fraction gave a major peak with a molecular mass of 16.25 kDa **(Figure 3.3).** According to these results the active enzyme is monomeric in nature.

This is the first report of the complete purification and characterization of smallest lipase with molecular mass of 16.25 kDa when compared with lipases purified to homogeneity from fungal origin reported till today and from different *Rhizopus* species so far studied [Hiol, et. al., 2000; Iwai and Tsujisaka 1974; Diaz , et. al., 2006; Sun, et. al., 2009]. Low molecular weight enzyme offers relatively easy modification of amino acids which are involved in active site by side directed mutagenesis. This can be exploited to get wider and better substrate specificity. Isoelectric point of the enzyme was determined to be 4.2 by isoelectric focusing as described in materials and method section.

Table 3.1: Purification summary of lipase from *Rhizopus* strain JK-1.

Steps of Purification	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification Fold	Yield (%)
Culture broth	119556	55	2173	1.0	100
Ammonium sulphate fraction	79704	10	7970	3.67	66.66
Dialyzed fraction	39852	3.42	11652	5.36	33.33
Q-Sepharose Chromatography I	30853	2.09	14762	6.79	25.80
Q-Sepharose chromatography II	27942	1.5	18628	8.57	23.37

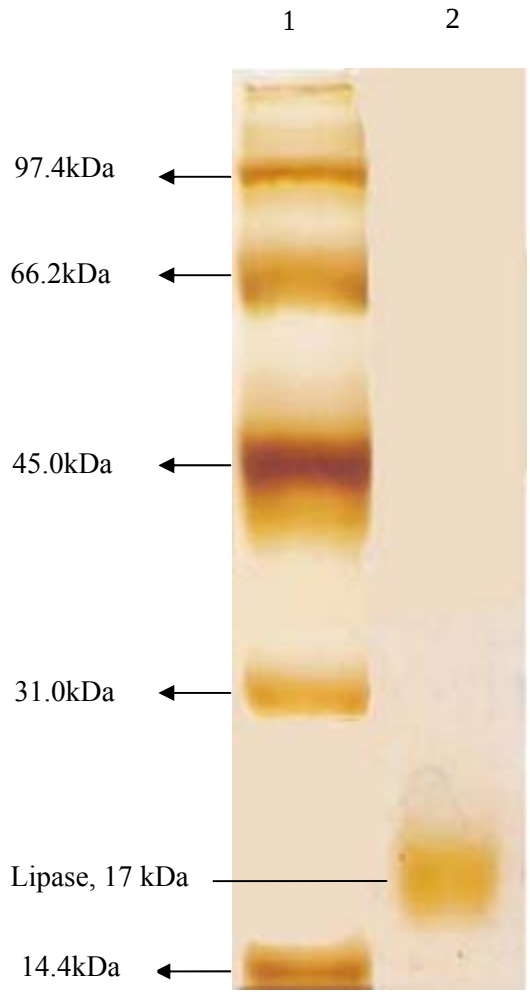


Figure 3.1. SDS–PAGE analysis of purified *Rhizopus* JK-1 lipase. Lane 1: molecular weight markers; lane 2: purified lipase.

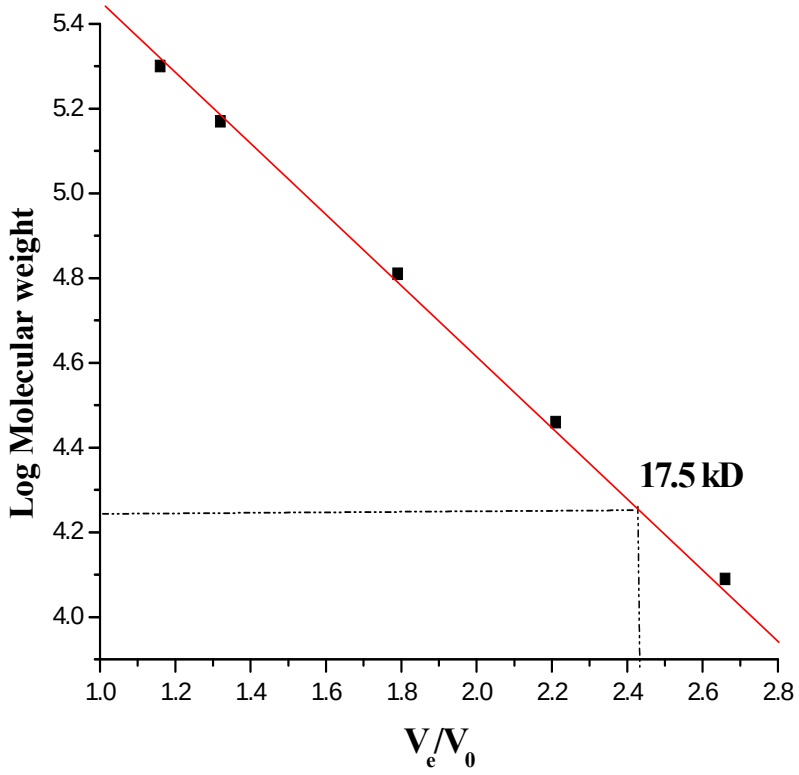


Figure 3.2. Molecular weight estimation of purified lipase from *Rhizopus* strain JK-1 by gel filtration on Sephadex G-200.

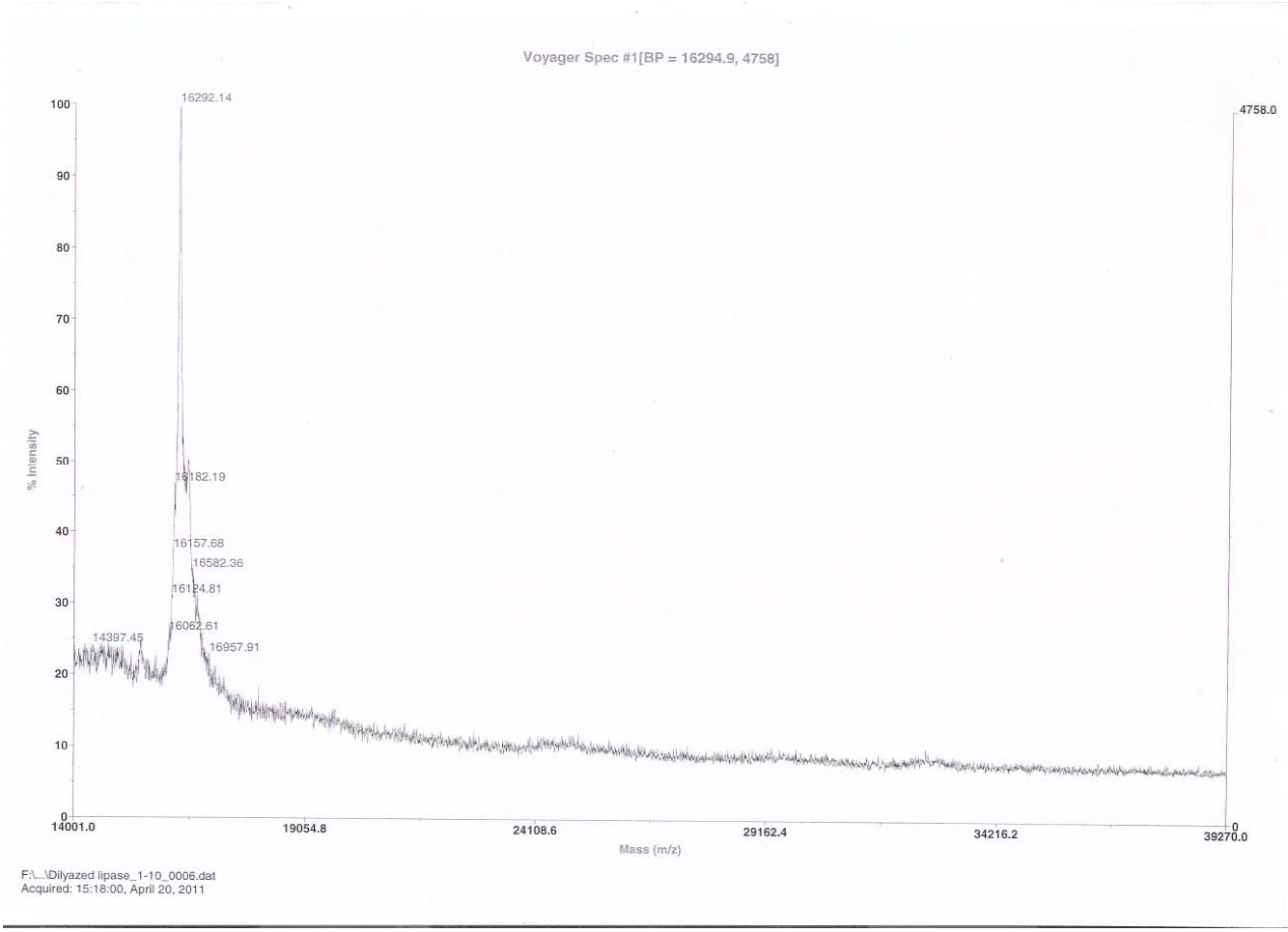


Figure 3.3. MALDI-TOF MS spectrum of purified Lipase from *Rhizopus* strain JK-1.

3.3.2. Effect of temperature and pH on lipase activity / stability

Studies on effect of temperature on lipase activity showed that the enzyme was active in the range of 30 °C-50 °C with optimum temperature at 40 °C **(Fig 4 A).** The activity dropped sharply beyond 50 °C. As, at high temperatures denaturing of enzyme shows loss of activity and at lower temperatures due to low activation energy reduces the activity of the enzyme. We report 40 °C as optimum temperature required for the activity of mesophilic *Rhizopus* strain JK-1, however thermophilic *R. oryzae* showed optimum temperature activity at 30 °C and enzyme was inactivated at 45°C seems from thermostability studies that the enzyme is highly inactivated at 45°C and that almost all activity is lost at 50°C after 30 min incubation [Hiol, et. al., 2000]. Similar profile was observed for lipase from *Rhizopus chinensis* [Sun, et. al. 2009].

Temperature stability profile of the enzyme under study shown in **Figure 4.B.** Lipase from *Rhizopus* strain JK-1 showed 95-100 % stability when incubated at 50 °C for 1 h. Almost 75 % activity could be recovered after incubating at 50 °C for 2 h, which dropped further to 60 % after 3 h incubation. Similarly when enzyme was incubated at 40 °C for 3 h, almost 81 % activity was retained. ThermosTable activity of present lipase is quiet promising when it is required to carry out reactions at higher temperatures for longer time. Among lipases purified to homogeneity from *Rhizopus* species, *Rhizopus* strain JK-1 lipase showed better thermostability [Hiol et. al., 2000; Iwai and Tsujisaka 1974; Diaz , et. al., 2006; Sun, et. al., 2009]. At 60 °C, 85 % loss of activity was seen after 1h incubation. Similar profile was observed for lipase from *Rhizopus chinensis* maximum activity was observed at 40 °C after 60m of incubation but the activity was reduced to 55% after 60 min at 50 °C and activity decreased dramatically when the temperature increased above 60 °C [Sun, et. al., 2009].

Studies on the effect of pH on enzyme catalysis showed maximum activity of the enzyme at pH 7.0 (**Figure 4 C**), with a narrow active pH range of 6.5 to 7.5. Activity dropped rapidly above pH 8.5 with only about 10-11 % residual activity at pH 9.0 was observed. According to the literature review different pH optima values for the lipase activity were reported such as pH 5.6 for *A. niger* [Fukumoto, et. al. 1962], pH 8.0 for that of *Humicola lunuginosa* [Liu, et. al., 1973] and enzyme purified from *Mucor* sp. showed maximal activity at pH 9.0 [Nagaoka K, Yamada Y. 1973]. *Rhizopus* species so far studied have very small pH optima range 7-7.5 [Hiol et. al., 2000; Sun, et. al., 2009; Kermasha, et. al., 1998], the lipase understudy is having the similar pH property as of these species reported earlier.

pH stability studies showed similar profile as of pH optima **(Figure 4 D).** Enzyme incubated at pH 6.0 for an hour retained almost 70 % activity, whereas at pH 7.0 more than 90 % activity was retained after 1 h incubation. There was 65 % loss of activity when enzyme was incubated at pH 8.0 and 5.0 for an hour.

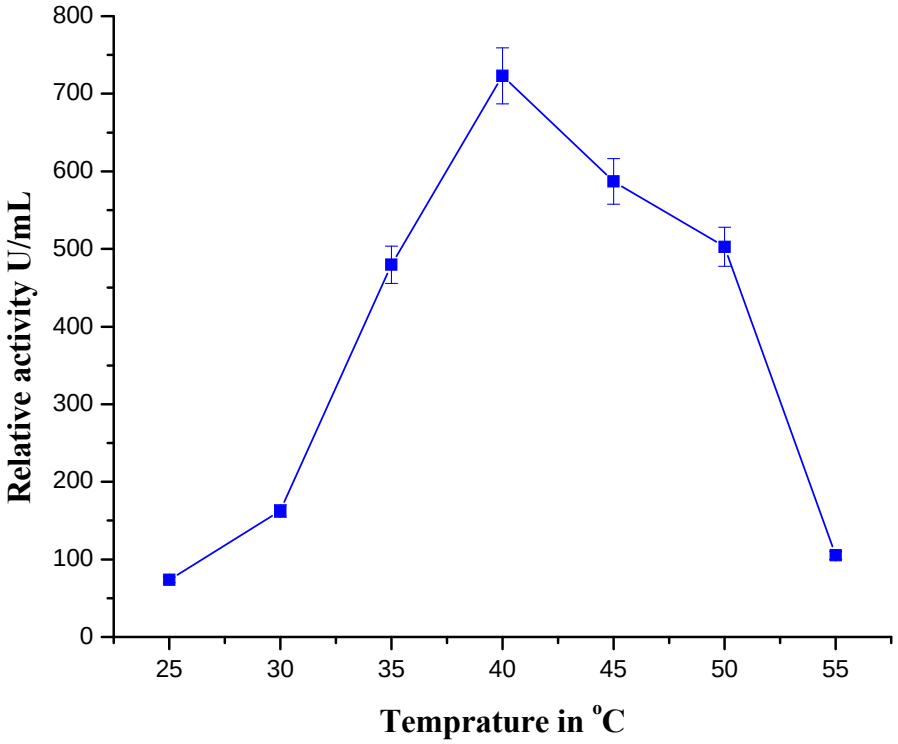


Figure 3.4. Effect of temperature on enzyme action when incubated at different temperatures ranging from 25-55 ºC.

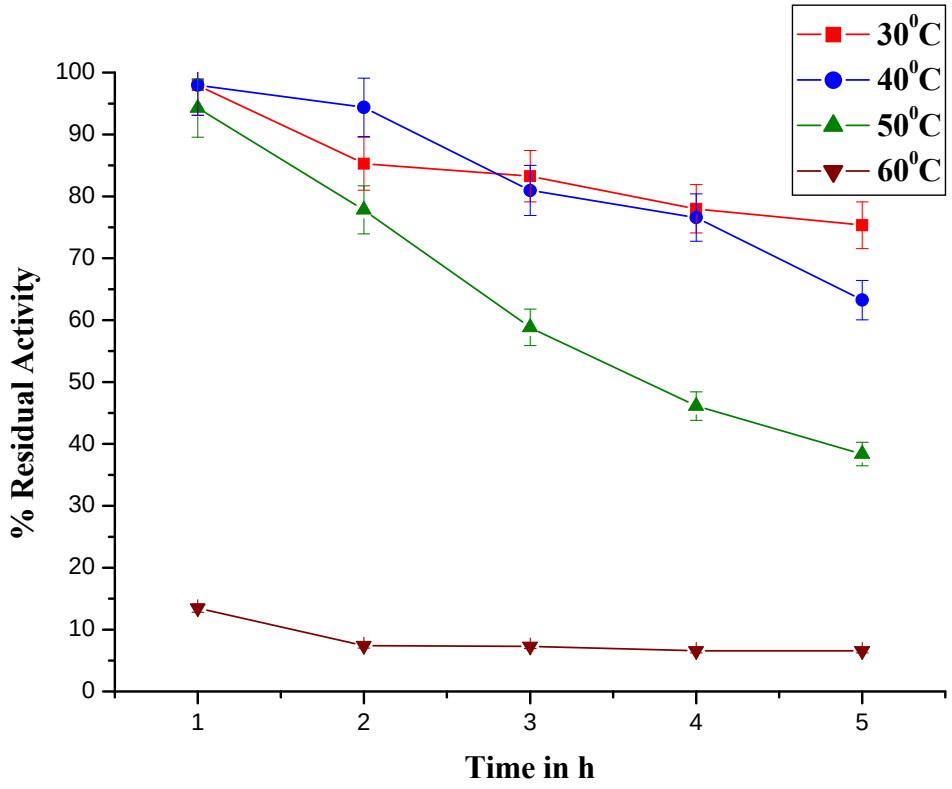


Figure 3.5. Lipase stability at different temperatures for 1-5 h. Enzyme stability was studied by measuring activity after 1h interval incubation at temperature ranging from 30 °C to 60 °C. All the values are average of assays carried out in triplicate.

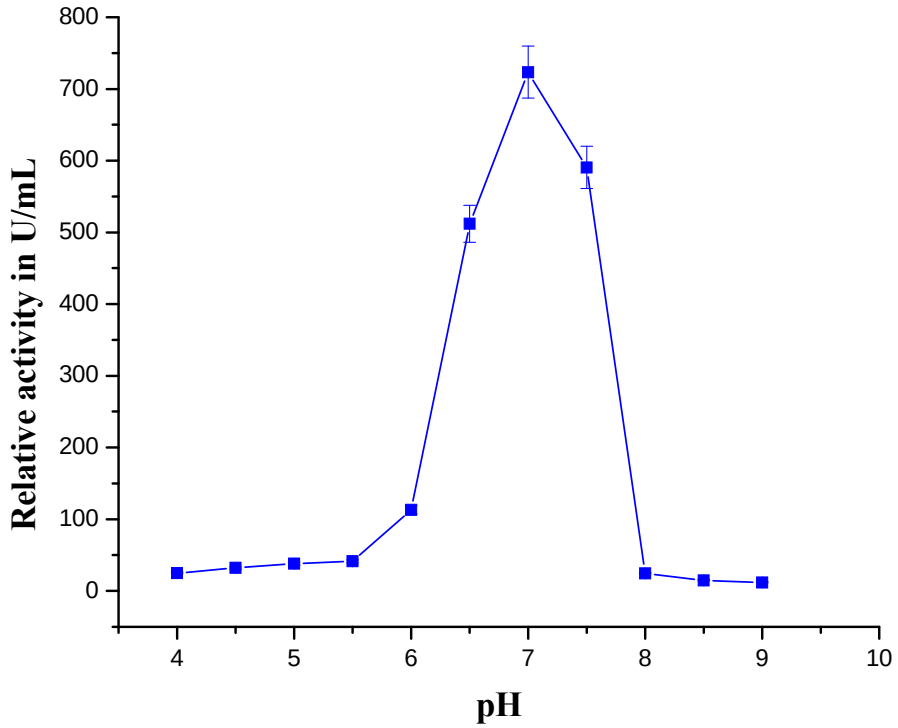


Figure 3.6. Effect of pH on lipase activity, purified enzyme was assayed with different pH buffers ranging from 4.0- 10.0.

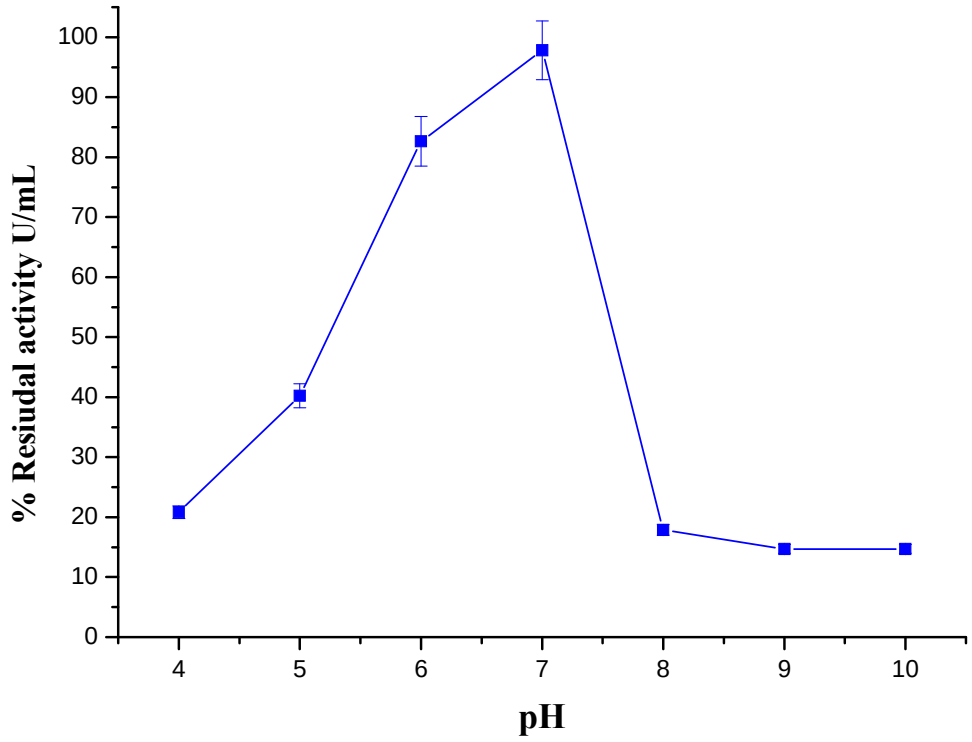


Figure 3.7. The effect of pH on enzyme stability was studied by measuring residual activity after one-hour incubation at pH ranging from 4.0 to 10.0. Activity is expressed as percentage of activity determined at pH 7.0. Values are means ± SD (n = 3).

3.3.3. Effect of various metal ions and amino acid modifying reagents on lipase activity:

The effect of metal ions and enzyme inhibitors are summarized in **Table 2 and 3**. Lipase activity was strongly inhibited by Hg^{++}, Cu^{++}, Fe^{++} and Zn^{++} which are in accordance with results reported about *R. oryzae* [Hoil, et. al., 2000]. Also strong inhibition was observed with Ba^{++}, Ag^{++} and Ni^{++} and very less inhibition (25 %) was observed with Co^{++} at 10mm concentration. The enzyme activity was not affected in presence of Mn^{++}, Ca^{++} and Mg^{++} (10mm) rather there was marginal increase in the enzyme activity. Similar pattern was observed in lipases reported from *Mucor hiemalis f. hiemalis* [Hoil,et. al., 1999], *Yarrowia lipolytica* [Yu, et. al.,2007], *Aspergillus carneus* [Saxena, et. al.,2003] when the enzyme was incubated with these divalent metal ions in concentration of 10 mM.

In active site characterization N-Bromosuccinamide strongly inhibited the enzyme activity at a concentration of 50 μM after 30m, with residual lipase activity of only 20 %. Strong inhibition due to N-Bromosuccinamide suggested that the active site of the enzyme under study may have tryptophan at or near its active site. More than 50-60 % activity was retained in presence of PMSF (Phenyl methyl sulphonyl fluoride) when 1mM concentration was used and Woodwards reagent k (2mM) after incubation for 30m. Lipases are generally members of the serine hydrolyses, with serine as an essential residue for catalytic activity [Salameh and Wiegel, 2007]. Incubation of enzyme with PMSF showed about 40 % inhibition. According to the reports and comments of Ateslier ZBB and Metin K [Ateslier and Metin, 2006] present lipase may belongs to the class of serine hydrolyses. Only 40 % loss of activity suggests that there might be the special domain (lid), making PMSF difficult to access the enzyme active site. There was no

inhibition observed with modifying agents such as DTNB (5, 5′- dithiobis- 2-nitrobenzoic acid) at 1mM concentration and TNBS (Trinitrobenzene sulphonic acid) at 25mM concentration for 30m, indicating cystein and lysine are not involved in the active site.

Table 3. 2: Influence of metal ions on the lipase activity when incubated with 10mM respective metal ions for 1 h. Activity without metal ion was considered as 100 %. Values are means ± SD (*n* = 3).

Metal Ions	% Residual Activity
Cu^{++}	0.00
Ba^{++}	24.34 ±1.16
Ag^{++}	21.25 ± 0.95
Hg^{++}	2.49 ± 0.39
Co^{++}	75.05± 3.01
Ni^{++}	24.57 ±1.32
Mn^{++}	99.85 ±1.91
Zn^{++}	0.00
Mg^{++}	101.33 ±1.87
Fe^{++}	0.00
Ca^{++}	100.5 ±0.48

Table 3.3: Effect of amino acid modifying reagents on lipase activity. Activity without inhibitor was considered as 100 %. Experiments were performed in triplicate and mean values are reported. Values are means ± SD (n = 3).

Amino acid modifier	Possible residue modified	Concentration	% Residual Activity
PMSF	Serine	1 mM	57.75 ± 1.02
DTNB	Cystein	1 mM	100 ± 0.89
Woodwards reagent k	Aspartic acid, Glutamic acid	2 mM	62.33± 2.59
NBS	Tryptophan	50 µM	21.60 ± 1.09
N-Acetyl imidazole	Histidine, Tyrosine	10 mM	86.41 ± 1.73
Citraconic anhydride	Lysine	25 mM	72.72 ± 1.24
TNBS	Lysine	25 mM	100 ± 1.48
Phenyl glyoxal	Arginine	3 mM	89.74 ± 0.80

3.3.4. Stability of lipase in different organic solvents

Lipase under study showed remarkable stability in organic solvents such as short chain alcohols like ethanol and methanol **(Table 3.4).** Incubation with 30 % ethanol showed loss of only 13 % of activity after 2 h incubation whereas with similar concentration of methanol 40 % loss of activity was observed after 2 h. These results are in contrast with the results obtained by lipase from *R. oryze* where lipase is not active in short chain (C1–C3) alcohol [Fariha, et. al., 2006]. This property can explore the potential of present lipase to be used as a biocatalyst for reactions such as transesterification or chiral resolution in organic reactions. It was also observed that lipase is not sTable in acetone and similar results were repored by Hoil et. al., in 2000. Whereas enzyme is not sTable in cyclohexane, but *Rhizopus chinensis* [Sun, et. al., 2009] and *R. oryzae* [Hoil, et. al., 2000] enzyme was very sTable in Cyclohexane and about 80% activity was retained in hexane.

Table 3.4 : Stability of *Rhizopus JK-1* lipase in organic solvents. Lipase when incubated in different organic solvents (30 % v/v) at 40 °C for 2 h. Residual activity was determined and expressed as percentage of the activity of the enzyme sample prepared in 50 mM phosphate buffer in absence of organic solvents. Activity without any organic solvent was considered as 100 %. Values are means ± SD (n = 3).

Organic Solvents	% Residual Activity
Methanol	62.99 ± 0.74
Ethanol	87.63 ± 0.68
Propanol	24.59 ± 1.38
Butanol	8.24 ± 0.28
Acetone	14.54 ± 1.17
Hexane	79.63 ± 0.99
Cyclohexane	38.74 ± 1.91

3.3.5. Enzyme-Catalyzed Transesterification of Oleic Acid

According to the solvent stability studies of the present enzyme, it was observed that the enzyme was sTable in ethanol than methanol; therefore, ethanol was used for transesterification reaction. Transesterification of oleic acid with the purified lipase showed about 66% conversion according to the FID counts compared with the standard ethyl oleate. Maximum ethyl ester production was observed at 24 h. Chromobacterium viscosum lipase when used in free form showed 62% ester formation at 8 h, which increased up to 71% when immobilized enzyme was, used [Shah, et. al., 2004]. Further work is underway to optimize the transesterification parameters to maximize the ethyl esters yield.

3.4. Conclusion:

Newly isolated *Rhizopus* strain JK 1 produces extracellular lipase which was purified as smallest monomeric lipase has been purified to homogeneity from new source of *Rhizopus* strain JK-1. The purified enzyme exhibited important properties in particular, optimum temperature for lipase action was 40 °C and enzyme showed remarkable thermostability when compared with other lipases reported so far from *Rhizopus* species. Optimum pH and pH stability profile of present lipase were observed to be similar to that of reported earlier. In presence of metal ions like Mn^{++}, Ca^{++} and Mg^{++} marginal increase in the enzyme activity was observed and in presence of Cu^{++} Fe^{++} Zn^{++} complete loss of activity was observed. N-Bromosuccinamide strongly inhibited the enzyme activity suggesting that tryptophan may be at or near its active site. We could show that the present lipase is suitable in short chain alcohols such as ethanol and methanol in 30% v/v concentration, therefore could be a good candidate for

transesterification reaction. Purified lipase showed good conversion of oleic acid with ethanol (when used in proportion of 1:4) by transesterification.

Chapters IV

Transesterification of Jatropha and *Pongamia* oil

Part A: Transesterification of Jatropha and *Pongamia* oil using purified enzyme

Abstract

Transesterification of *Pongamia* and *Jatropha* oil was carried out by using extracellular lipase enzyme obtained from, *Rhizopus* strain JK-1 with ethanol. Among the alcohol tested, ethanol gave maximum conversions when compared with the methanol. Different parameters like temperature, time and oil to alcohol ratio were studied and optimized for transesterification reaction. Maximum conversion of oil in their respective ethyl ester was observed after 8h of reaction, using oil and ethanol in 1:4 ratio. Ethyl esters yield reaches up to 73.90 and 71.20% from *Pongamia* and Jatropha oil after incubation for 8h at 30°C.

4a.1. Introduction

Biodiesel is derived from triglycerides of oil or free fatty acids by the processes of transesterification or esterification with short chain alcohols. There is renewed interest and increased awareness in researching alternative energy sources such as biodiesel for use in diesel engines when there is heavy dependency of current world on petro-diesel or fossil fuel and limited availability. Due to viscous nature of oil, it shows low ignition property (cetane number) and cannot be used directly in the engines like Petro-diesel [Openshaw, 2000]. This problem can be solved by transesterifying the oil to alkyl esters, which will subsequently reduce the viscosity and cetane number. Transesterification reaction takes place in presences of alcohol and catalyst, which can be either chemical or biological. In chemical catalysis either alkali or acids are used depending on the desired end-product. Different acids which can be used as catalysts are sulfuric, phosphoric, hydrochloric and organic sulfonic acids, but these catalysts require higher temperature and longer reaction times. Alkali based transesterification includes NaOH, KOH, carbonates and different alkoxides such as sodium methoxide, sodium peroxide, etc.

(Fukuda et. al. 2001 and Ma, 1999). Main disadvantage in using alkali is when higher free fatty acid (FFA) content is high. This requires excess amount of alkali which causes loss of FFA as insoluble soaps. Due to this final yield of esters decreases and alkali consumption increases. This leads to several disadvantages like increase in viscosity, formation of gels and recovery of glycerol becomes difficult [S. Shah et. al., 2004].

Considering these drawbacks of chemical catalysis, enzyme based biological catalyst showed favourable properties in terms of recovery, ambient temperature, reaction with high number of fatty acids (Fukuda et. al., 2001; Shah, et. al.,2004; Ban et. al 2001 and Modi, et. al., 2006 ; 2007 etc.). In biological catalysis, enzyme is used in either immobilized form or in free form for production of biodiesel in presence of alcohol. As per literature and reports, 80% yield could be obtained through transesterification with lipase from *M. miehi, Candida antarctica, Pseudomonas cepacia* by using primary alcohols such as methanol, ethanol or propanol etc. (Fukuda, et. al., 2001). Shah, et. al., in 2004 have reported enzyme based transesterification of Jatropha oil using three different lipases obtained from *Chromabacterium viscosum, Candida rugosa,* and Porcine pancreas in solvent free system. The yield for ethyl ester obtained was 73%. Another report from Modi, et. al., 2006 and 2007 illustrated transesterification using immobilized *C. antartica* lipase B. In this report they used Jatropha and *Pongamia* oil with propan-2- ol and ethyl acetate as acyl acceptor. In this study oil to ethyl acetate molar ratio used was 1:11 and the reaction time is about 12 h. Where as in alcohol as acyl acceptor, oil to alcohol molar ratio was 1:4 for 8 h. Maximum yield of ethyl esters in both the cases was more than 90%. In both the cases maximum conversion was at 50°C

In present **Chapter** purified lipase from *Rhizopus* strain JK-1 (Kantak, et. al., 2011 and 2012) was used for transesterification of *Jatropha* and *Pongamia* oil. Transesterification

parameters such as time, temperature, alcohol type and oil to alcohol ratio were also studied and optimized.

4a.2. Materials and Methods

4a.2.1. Materials

Enzyme lipase was purified from new isolate *Rhizopus* strain JK-1 as described in **Chapter III** [Kantak & Prabhune, 2012]. Oleic acid methyl ester, Fatty acid methyl ester mix and heptadecanoic acid methyl ester (served as the internal standard) were purchased from Sigma and were chromatographically pure. *Jatropha* and *Pongamia* oil were purchased from local vendors. All other chemicals were obtained commercially and were of analytical grade.

4a.2.2 Microorganism

Rhizopus strain *JK 1* was maintained on MGYP (Malt Extract- 0.3%, Yeast Extract- 0.3%, Peptone- 0.5%, Glucose-2% and Agar 2%) medium slants stored at 4°C and subcultured every 15-30 days. 10 ml sterile medium was inoculated with spores (approximately 10^6/ml) from fresh agar slant culture and incubated for 48 hr at 30°C and 180 rpm. This seed culture was used as inoculum for fermentation medium throughout the studies.

4a.2.2. Methods

4a.2.2.1. Enzyme Purification

Extracellular lipase from *Rhizopus* strain JK-1 was purified to the homogeneity through ion exchange chromatography as described in **Chapter III [**J. B. Kantak & A. A. Prabhune, 2012]. 72h grown culture broth was loaded on the freshly packed Q-

sepharose column and elution was done with 50mM phosphate buffer pH 7.0. Active fractions were pooled, concentrated and rechromatogramed on freshly packed Q-sepharose column. Purity of fractions were checked by SDS- PAGE. Active fractions with single band were used as source of lipase enzyme for transesterification**.** Protein concentration of purified enzyme after purification was 1.5mg/ml with specific activity 18,628 U/ mg.

4a.2.2.2. Effects of oil to alcohol ratio on transesterification of *Jatropha* and *Pongamia* oil:

Optimum concentration of alcohol leads to the maximum conversion of triglycerides to alkyl esters as alcohol is acyl acceptor in transesterification reaction. To determine the best oil to alcohol ratio for the transesterification of *Pongamia* and *Jatropha* oil, transesterification reactions were carried out at different oil to alcohol ratios such as 1:2, 1:4 and 1:6. Flasks were incubated at 30°C at 100rpm. Transesterification was carried out using relatively low shaking speed to avoid evaporation loss evaporation of alcohol added. Out of different rpm tested 100rpm gave optimum ethyl ester production, hence same shaking speed was continued throughout the studies. After 30 min of incubation, purified lipase (900 U) diluted in 0.5mL of 50mM phosphate buffer pH 7.0 was added to catalyse the transesterification reaction. The progress of the reaction was monitored by removing aliquots (100 μL) at various time intervals up to 48 h. The samples was centrifuged and diluted in n-hexane and used for the ethyl ester analysis by gas chromatograph with flame ionization detector (Master GC DANI, Italy). All experiments were done in triplicates and results were mean of three observations.

4a.2.2.3. Gas chromatography (GC) analysis

Ethyl esters in the reaction mixture were analysed by Gas Chromatograph with Flame – ionization detector (Master GC DANI, Italy). The capillary column (HP-INNOWax, Agilent Technology, USA) used had a length of 30 m with internal diameter of 0.25mm. Nitrogen was used as the carrier gas at a constant flow rate of 4 kg cm^{-2}. The column oven temperature was programmed from 80 to 210°C (at the rate of 50 °C/m) and was held at this temperature for 6 min. The oven temperature is further increased to 230°C (at the rate of 5 °C/ min.) with same temperature hold for 8 min. The injector and detector temperatures were 230 and 240 °C respectively.

4a.2.2.4. Effect of time on transesterification of *Jatropha* and *Pongamia* oil:

To study effect of time on transesterification of *Jatropha* and *Pongamia* oil reaction was carried out in 25-ml stoppered bottle up to 48h under shaking condition (100 rpm) at 30°C. Rest of the reaction conditions were same as described in section 4.2.2.1. Samples were removed after completion of 2, 4, 8, 12 and 24h. Samples were centrifuged and diluted with n-hexane and used for the ethyl ester analysis by gas chromatograph with flame ionization detector.

4a.2.2.5. Effect of ethanol and methanol on transesterification of *Jatropha* and *Pongamia* oil:

As per the reports available on enzyme transesterification, methanol has been used by maximum researchers. To evaluate better acyl acceptor between ethanol and methanol, two different reactions were carried out in two different stoppered bottles. Reaction mixture consisted of oil either *Pongamia* or *Jatropha* respectively and the ratio of oil to alcohol was kept as 1:4. Stopper bottles were incubated up to 48h under shaking condition (100 rpm) at 30 °C. Rest of the reaction parameters were same as described in section 4.2.2.1.

4a.2.2.6. Effect of incubation temperature on transesterification of *Jatropha and Pongamia* oil:

Temperature is a prime important parameter to be optimized for every enzymatic reaction. To determine the optimum temperature for the transesterification of *Pongamia* and *Jatropha* oil by purified lipase, reaction was carried out at different temperatures ranging from 25- 45°C at 100rpm. Samples were removed at specific time interval as described earlier in section **4a.2.2.3**. Samples were centrifuged and diluted with n-hexane. Ethyl ester analysis was done by gas chromatography with flame ionization detector as described in section 4a.2.2.1.

4a.3. Results and discussion

Considering the huge demand of industrially important enzyme lipase, there is continues need to investigate novel sources of lipases and their applications in transesterification. Sanchez and P Vasudevan in 2006 reported transetsrification of olive oil with *Candida antarctica* lipase with 91 % conversion. In another report Lee et. al., in 2009 transetsrification of olive oil, Soybean, palm, rapeseed, and sunflower oils with 85% conversion. R.C. Rodrigues, in 2009 studied transesterification of soybean oil with lipase from *Thermomyces lanuginosus* and many more previous reports where discussed by Fukuda, et. al., in 2001 for transesterification of vegetable oils. Transesterification of *Pongamia and Jatropha* oil with different types of acyl acceptors have been reported through the lipase from different sources such as *Chromobacterium viscosum*, *Candida rugosa,* Porcine pancreas and C*andida antarctica* [Shah et. al., 2004; Modi et. al., 2006 and 2007].

4a.3.1. Effects of oil to alcohol ratio on transesterification of *Jatropha* and *Pongamia* oil:

The yield of biodiesel products through lipase catalysis is modulated by the various parameters among which substrate to alcohol ratio (oil to alcohol) is prime important. In present study it was observed that maximum ethyl ester production (65.56% in *Pongamia* oil and 64% in Jatropha oil) achieved when oil to alcohol ratio was 1:4 (**Figure 4a.1).** When oil to ethanol ratio was 1:2 only 25% ethyl esters where produced, also increased amount of ethanol than 1:4 ratio didn't showed any increase in percentage transesterification yield. 1:6 ratio of oil to ethanol gave up to 45% ethyl ester production after 8h of reaction time. Similar ratio of alcohol to oil was used by Dong Hong Lee et. al., in 2006 for transesterification of soybean oil with the help of lipase. It was reported earlier that enzyme mediated transesterification of *Jatropha* oil also required 1:4 molar ratio of oil to ethanol [Shah, et. al., 2004].

Casimir, et. al., in 2007 showed that high concentrations or the addition of the required ratio of oil to methanol all at once has an inhibitory effect on lipase mediated transesterification. Addressing this toxic effect of methanol standardization of addition of alcohol (in this case ethanol) was attempted so as to get maximum efficiency. The alcohol required in this case 1:4 was added in two subsequent steps.

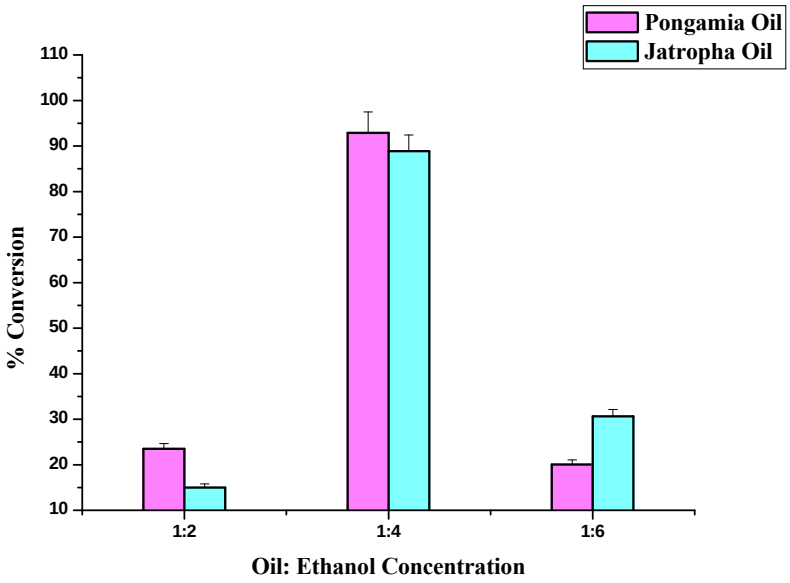


Figure 4a.1. Effect of oil to ethanol ratio on transesterification of *Pongamia* and *Jatropha* oil by purified enzyme.

4a.3.2. Effect of time on transesterification of *Jatropha* and *Pongamia* oil

Figure 4.2. shows the progress of transesterification reaction of *Jatropha* and *Pongamia* oil along with time. Maximum ethyl esters yield (72 %) was achieved after completion of 8h reaction. For both Jatropha and *Pongamia* oil 8h reaction time gave maximum yield (73% from *Pongamia* oil and 71% from *Jatropha* oil), which remained constant thereafter. Similar observations were noted by Modi, et. al., 2007, maximum yield was achieved after 12h of reaction. In present study 900units of enzyme was used for each transesterification reaction where as Modi, et. al., 2007 used 10,000 units of enzyme.

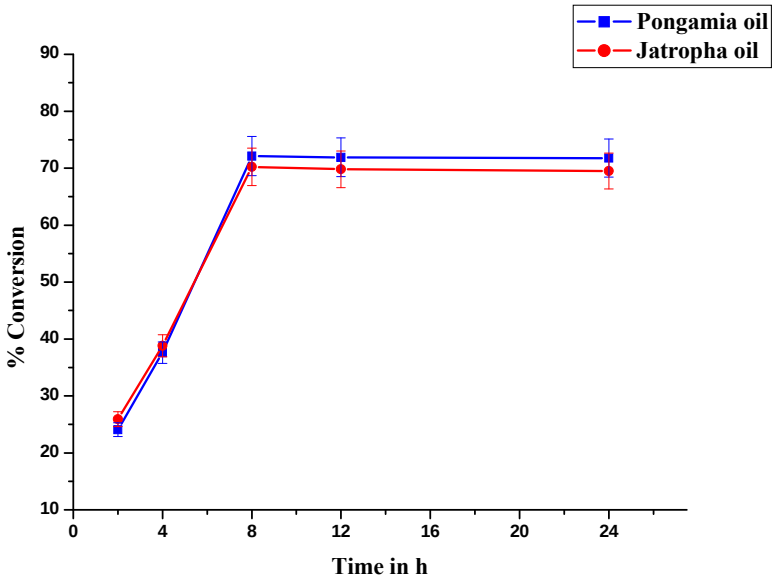


Figure 4a.2. Effect of time on transesterification of *Pongamia* and *Jatropha* oil by purified enzyme

4a.3.3. Effect of ethanol and methanol on transesterification of *Jatropha* and *Pongamia* oil:

Maximum conversion of both *Pongamia* and Jatropha oil was obtained in presence of ethanol as compared with that of methanol (**Figure 4a. 3 and 4a.4**). This is well justified on the basis of stability of present enzyme at relatively higher ethanol percentage. As described in **Chapter III** purified lipase was more sTable in ethanol (30%) than that of similar concentration of methanol (Kantak and Prabhune, 2012). In the above mentioned case purified lipase was sTable in 30% ethanol for 2h with loss of only 13% activity whereas in case of methanol 40 % loss of activity was observed. Shah, et. al., in 2004 used ethanol for transesterification of *Jatropha* oil and obtained 88% conversion when enzyme was immobilized. As per the report by Marchetti, et. al., in 2007 considering the toxic nature of methanol and its availability it's important to have a potent alcohol candidate like ethanol to achieve maximum conversion of triglycerides to fatty acid alkyl esters. Present enzyme is potent catalyst for transesterification of *Jatropha* and *Pongamia* oil which can use ethanol as acyl acceptor. Maximum conversion of both the oils with ethanol and methanol as acyl acceptors increased with time till up to 8h . After 8h of reaction there was no further increase in the ethyl ester yield. In case of ethanol about 73% *Pongamia* oil and 71 % Jatropha oil ethyl esters were produced. However in case of methanol as acyl acceptor yield does not increased beyond 35%.

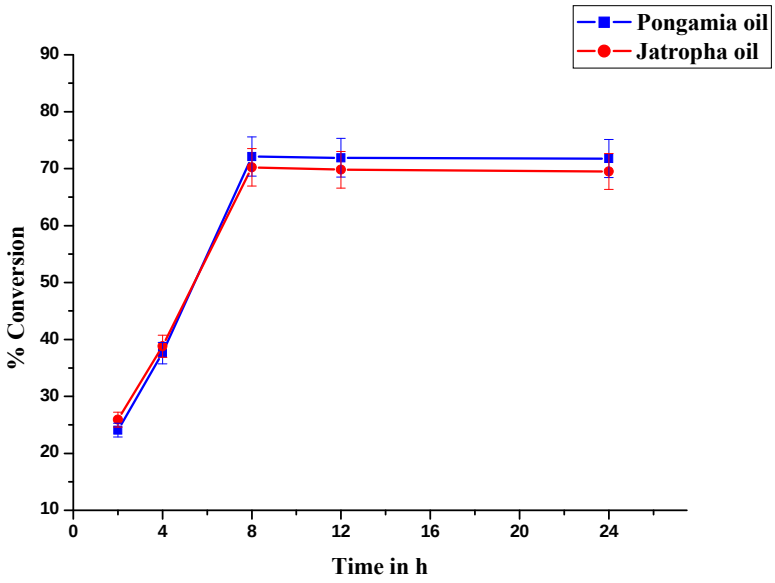


Figure 4a. 3. Effect of ethanol on transesterification of *Pongamia* and *Jatropha* oil by purified enzyme

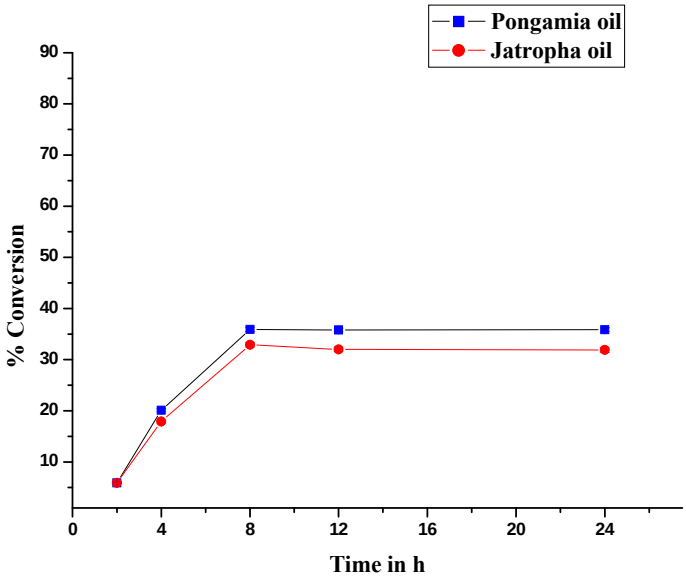


Figure 4a. 4. Effect of methanol on transesterification of *Pongamia* and *Jatropha* oil by purified enzyme

4a.3.4. Effects of incubation temperature on transesterification of *Jatropha* and *Pongamia* oil:

The very important parameter for any transesterification reaction is temperature and it should be ambient for obvious reasons. Previous report by Fukuda H. et. al., 2001 have shown formation of soaps from glycerids making the system inefficient. In present studies lipase from new source, *Rhizopus* strain JK-1 showed maximum ethyl ester production at 30°C. Earlier reports from Modi et. al., 2007; Lee, et. al., 2009; Sanchez and Vasudevan, 2006 achieved transesterification using free or immobilized enzyme but at higher temperature ranging from 40- 50°C. Using higher temperatures adds to the cost of transesterification**. Figure 4a.5** shoes the effect of temperature on transesterification, increase in temperature lowered the percentage of conversion. Maximum conversion was obtained at 30°C with transesterification parameters stated earlier. Transesterification yield goes decreasing as temperature increases (**Figure 4a.5**). At higher temperatures the transesterification rate drops, and only 25% ethyl esters were obtained at 40°C. Lower temperature optima for the present lipase offer it as a best suiTable candidate for transesterification of non edible oils.

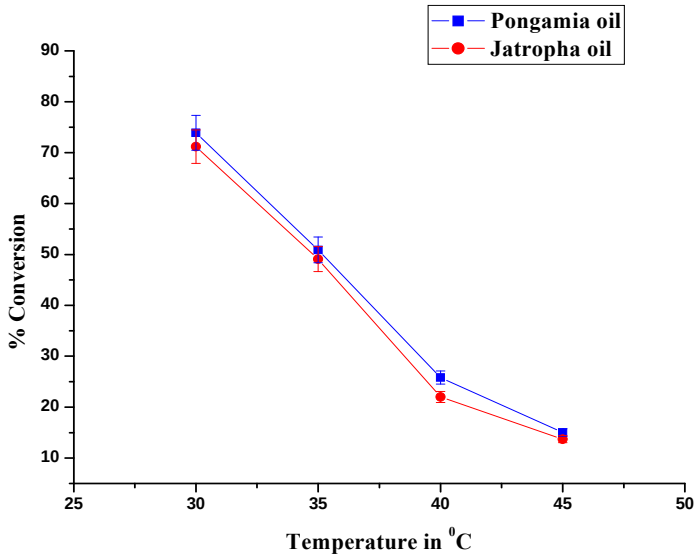


Figure 4a.5. Effect of temperature on transesterification of *Pongamia* and *Jatropha* oil by purified enzyme

Conclusion:

Present Chapter described the use of purified enzyme from novel source *Rhizopus* strain JK-1 for transesterification of *Pongamia* and *Jatropha* oil and optimization of parameters required for transesterification. Transesterification of non edible oils such as *Jatropha* and *Pongamia* has been done using ethanol as acyl acceptor. Transesterification reaction was catalysed by the lipase enzyme from new source *Rhizopus* strain JK-1. Different parameters require for maximum production of ethyl esters have been optimized. Maximum ethyl ester yield (73% from *Pongamia* oil was and 71% from Jatropha oil) achieved after 8h of reaction when 900units of purified enzyme was used for transesterification. The best ratio of oil to ethanol was 1:4 which gave optimum ethyl ester production at 30°C after 8h. Ethyl ester yield can be further increased by increasing the enzyme concentration and immobilizing the purified lipase which can be reused for several times.

Chapter IV:

Transesterification of *Jatropha* and *Pongamia* oil

Parts B: Transesterification of *Jatropha* and *Pongamia* oil using whole cells of *Rhizopus* strain JK-1

Abstract:

Transesterification of *Pongamia* and *Jatropha* oil was carried out by using extracellular lipase producing whole cells of novel isolate, *Rhizopus* strain JK-1 as biocatalyst. Ethanol gave maximum conversions when compared with the methanol as acyl acceptor. Ethanol gave up to 95% ethyl ester production from *Pongamia* oil after 36h of transesterification reaction. Different parameters like temperature, time and oil to alcohol ratio were standardized for optimum conversion. Maximum conversion of oil into ethyl ester was found at 30°C. Ethyl ester production increased with time and maximum production (95% from *Pongamia* oil and 88% from *Jatropha* oil) was achieved after completion of 36h of reaction at 30°C. Oil to ethanol concentration also affected the conversion rate in both the oils. Oil to ethanol concentration which gave best conversion was 1:4. In present **Chapter** successful transesterification of *Jatropha* and *Pongamia* oil with ethanol as acyl acceptor using extracellular lipase producing whole cells of *Rhizopus* stain JK-1 was carried out.

4b.1. Introduction:

Biodiesel consists of alkyl ester of fatty acids produced by transesterification of triglycerides (TGs) with primary alcohols (i.e. ethanol, methanol, etc). Biodiesel is a clean, renewable and domestically produced diesel fuel, which has many characteristics of a promising alternative energy source [Fukuda, et. al., 2001]. It is biodegradable and the combustion products have reduced levels of particulates, carbon monoxide, sulfur oxides, hydrocarbons, soot, and nitrogen oxides [Du, et. al., 2003 and 2004; Xu, et. al., 2004]. Number of processes has been developed for biodiesel production like chemical, enzyme catalysis and supercritical alcohol treatment although each process has drawbacks and advantages [Zhang, et. al., 2003]. Considering the several drawbacks of

chemical processes, enzymes are getting more importance. The various processes and chemicals involved in enzyme purification are cost incurring which is the main barrier for using enzymes as catalysts for esterification. Immobilized lipases in particular are suiTable for continuous biodiesel-fuel production because of its easy recovery from the reaction mixture and repeated reuses, making the processes cost effective. According to reports available, effective enzyme immobilization techniques such as use of silica gels extends lipase life without losing yield [Hsu, et. al., 2002]. However construction of such gels is a time consuming and expensive processes [Fukuda, et. al., 1996]. In addition, even with the ability to reuse the enzymes for several reaction cycles, the cost continues to be high if the matrix for immobilization is expensive. This can be controlled by using low cost and renewable supports. To reduce the cost further, utilization of lipase producing whole-cell as biocatalysts is significantly advantageous since no purification of the enzyme is necessary. Ban et. al., in 2001have demonstrated for the first time efficient catalysis of vegetable oil with the help of biomass supported particles (BSPs) of *R. oryzae* using methanol as acyl acceptors [Ban, et. al., 2001 and 2002]. Stepwise addition of methanol was recommended to minimize the negative effect of methanol on the activity of *R. oryzae* whole cells [Ban, et. al., 2001]. However, stability of the whole cells during repeated uses was poor. In order to increase the stability, *tert*-butanol was demonstrated as an ideal medium for biodiesel production, in which stability of the biocatalysts could be enhanced significantly [Wang, et.al., 2006; Wei, et. al., 2007].

In present **Chapter** attempts are made to minimize above mentioned drawbacks by using newly isolated extracellular lipase producing *Rhizopus* strain JK-1 whole cells in free form as biocatalyst for transesterification. The cost of oil source is another influencing factor on biodiesel. Production of biodiesel from refined vegetable oils is obviously not

sustainable because of cost and its end use. This can be replased by using relatively low cost non edible oils such as *Pongamia* and Jatropha. Biodiesel production from these two non-edible oils has been explored in present study using whole cells of *Rhizopus* JK-1. The transesterification reaction was affected by different parameters such as molar ratio of oil to alcohol, biocatalysts (whole cells), reaction temperature and reaction time. It is prime important to study all these variables to achieve maximum transesterification. In present **Chapter** optimum parameters which are required for maximum transesterification of both the oils by using lipase producing free whole cells were studied.

4b.2. Materials and methods

4b.2.1. Materials

Oleic acid methyl ester, Fatty acid methyl ester mix and heptadecanoic acid methyl ester (which served as the internal standard) were purchased from Sigma and were chromatographically pure. Corn steep liquor (CSL) was gift from Hindustan Antibiotics Ltd. Pune, India its pre-treatment was described in **Chapter II.** *Jatropha* and *Pongamia* oil were purchased from local market. All other chemicals were of analytical grade.

4b.2.2 Microorganism

Rhizopus strain *JK-1* was maintained on MGYP (Malt Extract- 0.3%, Yeast Extract- 0.3%, Peptone- 0.5% and Glucose-2%) medium slants stored at 4°C and subcultured every 15-30 days. 10 ml sterile medium was inoculated with spores (approximately 10^6/ml) from fresh agar slant culture and incubated for 48 hr at 30°C and 180 rpm. This seed culture was used throughout the studies.

4b.2.3. Methods

4b.2.3.1. Cells cultivation

Rhizopus strain JK-1 was grown under optimum culture conditions for production of extracellular lipase as described earlier in **Chapter III** [Kantak & Prabhune, 2012]. The organism was cultivated in 250 ml Erlenmeyer flasks containing 25 ml of double strength of basal medium (Glucose 2%, Na_2NO_3 0.2%, MgSO4 0.1, KH2PO4 0.2%, CSL 6%) seeded with 48h grown inoculum prepared in MGYP. Flasks were incubated in a rotary incubator for 72 h at 30 °C and 180 rpm.

4b.2.3.2. Gas chromatography (GC) analysis

Ethyl esters in the reaction mixture were analysed by Gas Chromatograph with Flame – ionization detector (Master GC DANI, Italy). The capillary column (HP-INNOWax, Agilent Technology, USA) used had a length of 30 m with a internal diameter of 0.25mm. Nitrogen was used as the carrier gas at a constant flow rate of 4 kg cm^{-2}. The column oven temperature was programmed from 80 to 210°C (at the rate of 50 °C/min) and hold at this temperature for 6min. The oven temperature is further increased to 230°C (at the rate of 5 °C/ min) with same temperature was held for 8min. The injector and detector temperatures were 230 and 240 °C respectively.

4b.2.3.3. Effects of oil to alcohol ratio on transesterification of *Jatropha* and *Pongamia* oil:

The transesterification reactions were carried out in 250ml Erlenmeyer flasks containing 72h grown extracellular lipase producing *Rhizopus strain JK-1* whole cells as described in section 4b.2.3.1. Reaction was started in the same flask without removing the mycelia by centrifugation or filtration. At 72h of growth, transesterification reaction was started by adding *Jatropha* and *Pongamia* oil (2g) and ethanol. Alcohol performs the role of the

acyl acceptor in transesterification reaction. Its prime important to optimize the concentration of alcohol for maximum conversion of triglycerides in to alkyl esters. To determine the oil to alcohol ratio for the transesterification of *Pongamia* and *Jatropha* oil, reaction was carried out at different combination such as 1:2, 1:4 and 1:6. Flasks were incubated at 30°C at 100rpm to minimizing the evaporation of alcohol during transesterification throughout the studies. To standardize Sampling and analysis was done as described earlier. The best ratio of oil to alcohol which gave maximum transesterification was used for further studies.

4b.2.2.4. Stepwise addition of ethanol:

It is well studied by Ban et. al., in 2001, that transesterification reaction was mainly affected by the amount of alcohol which is required for maximum conversion of triglycerides. To overcome this problem, stepwise addition of ethanol was standardized in present studies. The procedure giving optimum production was used for rest of the studies.

To optimize the stepwise addition of ethanol the transesterification reaction were carried out in 250ml Erlenmeyer flasks containing 72h grown (extracellular lipase producing) *R. oryzae* whole cells. At 72h of growth, transesterification reaction was started by adding *Jatropha* and *Pongamia* oil (2g) and ethanol (8g) maintaining the ratio of 1:4 in two different flasks, incubated at 30 °C and 100 rpm, rpm was kept relatively low for minimizing the evaporation of alcohol. Ethanol was added in two steps at two different time intervals such as at 0h, 6g of ethanol was added and remaining 2g at 24h to avoid negative effect. Two mL of sample was withdrawn from the reaction mixture after 12h time interval, centrifuged to obtain the upper layer and diluted with n-hexane, analyzed

by capillary gas chromatography. Every reaction was carried out in triplicates and readings were mean of three observations.

4b.2.2.5. Time kinetics of transesterification:

Rhizopus strain JK-1 cells were grown in optimized conditions as described earlier section 2.3.1. Two different sets of experiments were done for transesterification. In one set of transesterification, reaction was started after completion of 48h growth of *Rhizopus* strain JK-1 and in another set reaction was started after completion of 72h of growth of *Rhizopus* strain JK-1. Rest of the parameters were same as described earlier, including temperature and shaking. Samples were removed and analysis was done by Gas Chromatography with Flame –ionization detector.

4b.2.2.6. Effect of time on transesterification of Jatropha and *Pongamia* oil:

To optimize the time for maximum ethyl ester production, reaction was started after 72h growth of *Rhizopus* strain JK-1 cells as described earlier. In these flasks 2g of respective oil and 6g of ethanol was added and flasks were incubated at 30 °C and 100 rpm. Remaining 2g of ethanol was added after 24h, thereafter 2ml samples were aliquoted after each 12h interval up to 48h. Samples were centrifuged and upper layer was diluted in n-hexane. Analysis was done by Gas Chromatography.

4b.2.2.7. Effect of ethanol and methanol on transesterification of *Jatropha* and *Pongamia* oil:

To study the effect of ethanol and methanol on transesterification, reactions were carried out in 250ml flasks containing cells of *Rhizopus* strain JK-1 grown for 72h (where maximum production of enzyme was observed). Oil and alcohol were added in 1:4 ratio to start the reaction as described in earlier sections. Reaction was carried out in separate flasks for each oil. Flasks were incubated at 30°C and 100 rpm, again rpm kept low to

minimize the evaporation loss of alcohol. Samples were centrifuged and upper layer was diluted in n-hexane, analysis was done by Gas Chromatography.

4b.2.2.8. Effects of incubation temperature on transesterification of *Jatropha* and *Pongamia* oil:

Incubation temperature strongly influenced the transesterification reaction. Reaction was carried out in 250ml flasks containing 72h grown whole cells of *Rhizopus* strain JK-1 at 30°C. To determine the optimum temperature for the transesterification of *Pongamia* and *Jatropha* oil by whole cells, reaction was carried out at temperature ranging from 30- 45°C at 100rpm. Samples were centrifuged and upper layer was diluted in n-hexane, analysis was done by Gas Chromatography.

4b.3. Result and Discussion:

4b.3.1. Time kinetics of transesterification

As the reaction was carried out by the whole cells it is of prime importance to optimize the time (growth stage of the whole cells) to start the reaction at which maximum ethyl ester production can be achieved. Two different reactions were carried out to optimize the time to start the transesterification reaction. Reaction started after completion of 72h growth of *Rhizopus* strain JK-1 gave maximum ethyl ester production which was up to 95.0 % for *Pongamia* oil and about 89.0% for the *Jatropha* oil which took 36h after addition of oil. Whereas transesterification reaction initiated after 48h of incubation gave only 74% ethyl esters from *Pongamia* oil and 33% from Jatropha oil after completion of 36h of transesterification with no further increase. **Table 4b.1** shows the pattern of ethyl ester obtained in both the reactions which showed steady increase with time. Whereas sharp drop in ethyl esters percentage was seen after 48h. Similar pattern was seen when the transesterification reaction was initiated after 48h growth of

Rhizopus whole cells. Drop in transesterification yield after 48h can be attributed to the catabolic use of methyl esters by whole cells of *Rhizopus* strain JK-1.

Time in h	***Pongamia* oil**		***Jatropha* oil**	
	% Conversion		% Conversion	
	A	B	A	B
12	4.60	23.50	9.69	15.01
24	37.7	51.04	28.99	39.99
36	**73.96**	**94.45**	**32.86**	**88.80**
48	38.01	58.08	18.01	25.18

A- 48h grown culture and B – 72h grown culture.

Table 4b.1. Effect of initiation time on transesterification.

4b.3.2.Effects of oil to alcohol ratio on transesterification of *Jatropha* and *Pongamia* oil:

One of the most important variables affecting the yield of ester is the molar ratio of alcohol to triglyceride. As per the classical reaction of the transesterification a 1:3 molar ratio of alcohol to triglycerides is needed. In practice, the ratio needs to be higher to drive the equilibrium to a maximum ester yield (Ma and Hanna, 1999). Conversion of *Pongamia* and *Jatropha* oil required 1:4 ratio of oil to alcohol to yield the maximum ethyl esters in the present study **(Figure 4b.1)**. As depicted in the Figure 4b.1, 1:2 ratio of oil to alcohol gave only 20% conversion where as 1:4 gave maximum conversion up to 94%. In case of higher ratios like 1:6 conversion rate dropped and only up to 30 % ethyl esters were formed due to inactivation of lipase by excess of ethanol present in the reaction. Shah et. al., in 2004 reported the same ratio of alcohol to oil for esterification

of *Jatropha* oil. For maximum conversion of oleic acid in to ethyl oleate same ratio of oil to ethanol was reported earlier in previous studies (Kantak & Prabhune, 2012).

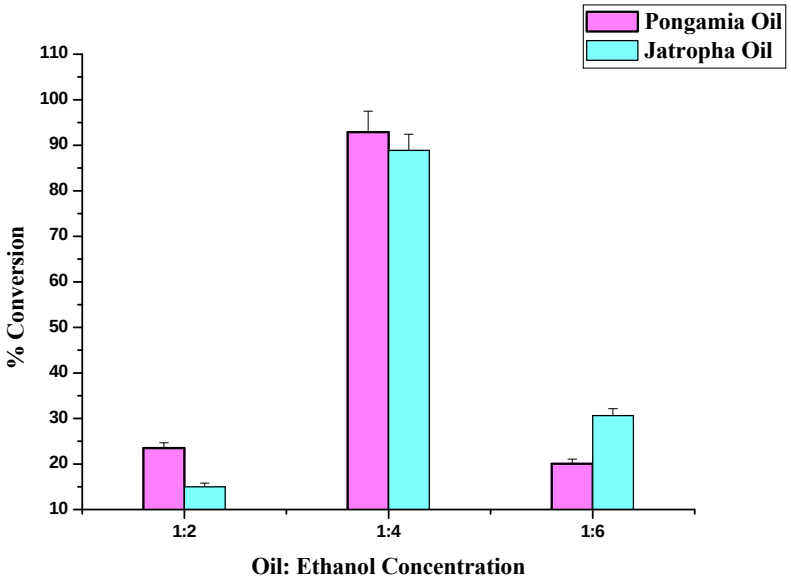


Figure 4b.1. Effect of oil to alcohol ratio on transesterification of *Jatropha* and *Pongamia* oil by whole cells of *Rhizopus* strain JK-1.

4b.3.3. Effect of time on transesterification of *Jatropha* and *Pongamia* oil:

Maximum ethyl ester production (95.0 and 90.0%) was obtained after completion of 36h of reaction at 30°C, 100rpm. **Figure 4b.2** shows the ethyl ester content in the reaction mixture along with time. *Pongamia* oil ethyl esters produced slightly higher in quantity than *Jatropha* oil ethyl esters. Phenolics compounds and other toxic substances like alkolides present in the *Jatropha* oil might have inhibited the enzyme action which is showen in case of Jatropha oil. Chen & Lin in, 2010 have reported growth accosiated transesterification of soybean oil using *R. oryzae* whole cells. We report 90-95% transesterification of Jatropha and Pongamia oils after 36h using pre-grown cells of Rhizopus.

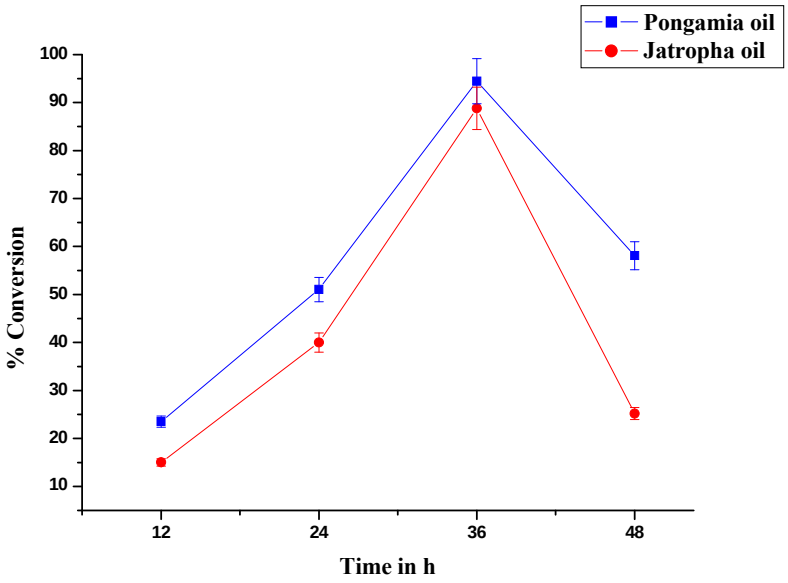


Figure 4b.2. Effect of time on transesterification of *Jatropha* and *Pongamia* oil by whole cells of *Rhizopus* strain JK-1. *Rhizopus* strain JK-1 was grown up to 72h at 30°C before starting the reaction.

4b.3.4. Effect of ethanol and methanol on transesterification of *Jatropha* and *Pongamia* oil:

To study the better acyl acceptor among ethanol and methanol two different reactions were carried out using respective alcohol. Ethanol gave the maximum conversion in case of both the oils (**Figure 4b.3)** as compared with the methanol **(Figure 4b.4).** When ethanol was used as acyl acceptor 95% of ethyl esters were formed from Pongamia. Considering the toxic nature of methanol and its availability it is important to have a potent acyl acceptor candidate to achieve maximum conversion of triglycerides to fatty acid alkyl esters [Marchetti, et. al., 2007]. As per the results described in previous **Chapters** lipase from the *Rhizopus* strain JK-1 shoed high stability in presence of ethanol as compared to methanol these results correlates with earlier reports of present lipase stability in organic solvents [Kantak and Prabhune, 2012]. These results are

contrary to the reports of Tamalampudi, et. al., in 2008 and Hama, et. al., in 2006 where methanol was better acyl acceptor for maximum production of esters.

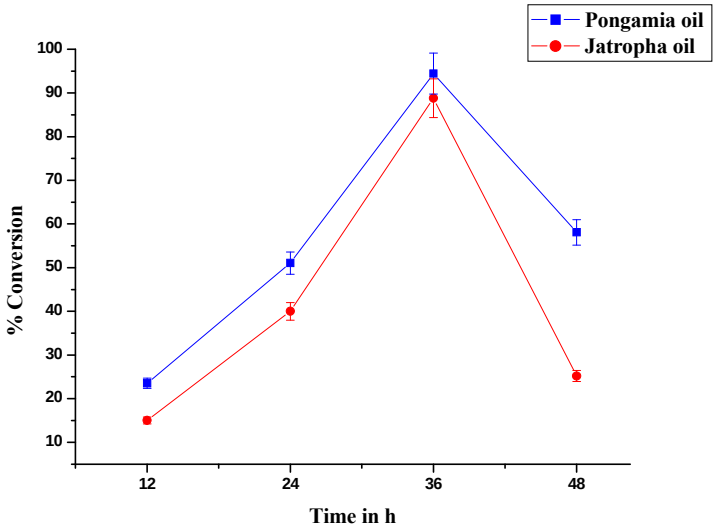


Figure 4b.3. Effect of ethanol on transesterification of *Pongamia* and *Jatropha* oil by whole cells of *Rhizopus* strain JK-1. *Rhizopus* strain JK-1 was grown up to 72h at 30°C before starting the reaction.

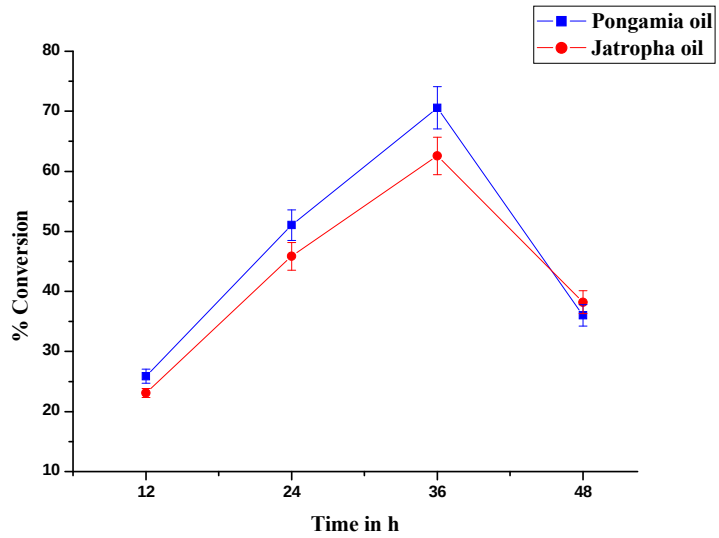


Figure 4b.4. Effect of Methanol on transesterification of *Pongamia* and *Jatropha* oil by whole cells. *Rhizopus* strain JK-1 was grown up to 72h at 30°C before starting the reaction.

4b.3.5. Effects of incubation temperature on transesterification of *Jatropha and Pongamia* oil:

Transesterification can occur at different temperatures, depending on the oil used and method applied, like chemical catalysis. Temperature clearly influenced the reaction rate and yield of esters in our case. Transesterification reaction carried out at different temperatures for both the oils and results are represented in the **Figure4b.5.** It was observed that reaction which was carried out at 30°C gave maximum fatty acid ethyl esters from both the oils. This is in agreement with our earlier reports (described in **Chapter III), where** 30°C was optimum temperature for maximum lipase production [Kantak, et. al. 2011]. These observations correlates with maximum production of lipase of *Rhizopus* strain JK-1. Sudden drop was observed after 35°C and only 30% ethyl esters were produced.

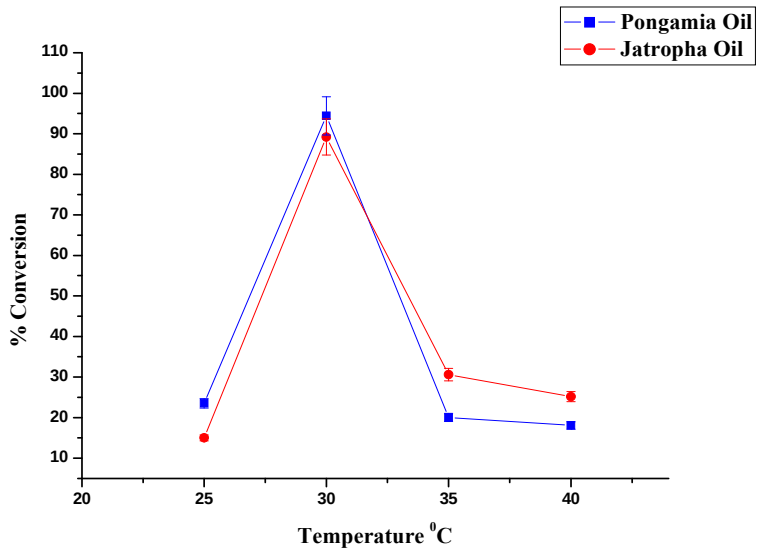


Figure 4b.5. Effect of temperature on transesterification of *Jatropha* and *Pongamia* oil by whole cells of *Rhizopus* strain JK-1. *Rhizopus* strain JK-1 was grown up to 72h at 30°C before starting the reaction.

Conclusion:

Transesterification of *Jatropha* and *Pongamia* oil was carried out successfully by using extracellular lipase producing whole cells of *Rhizopus* strain JK-1. Maximum amount of ethyl ester formed (95%) after 36h of reaction at 30°C. Ethanol was the best acyl acceptor in present transesterification reaction when compared with the methanol. Oil to ethanol ratio played important role in transesterification studies and maximum conversion of both the oils was carried out at 1:4 ratio of oil to ethanol.

Chapter V:

Simultaneous alcohol Fermentation and transesterification of *Jatropha* and *Pongamia* oil

Abstract

Transesterification of *Pongamia* and *Jatropha* oil was carried out by using whole cells of new isolate *Rhizopus* strain JK1 which produces extracellular lipase as described in Chapter 4B. In this study alcohol required for the transesterification of both *Pongamia* and *Jatropha* oil was made available using *Saccharomyces cerevisiae* by fermenting the simple sugars like glucose and sucrose. In transesterification reaction, the required ratio of oil to alcohol was found to be 1:4. The alcohol required for transesterification was produced using immobilized yeast cells with suitable substrate. The parameters were maintained in such a way so as to get the transesterification by alcohol production. Optimization of different parameters required for simultaneous alcohol production and transesterification was studied in detail. Maximum conversion of oil to ethyl esters (97% in *Pongamia* and 95 % in *Jatropha*) was observed when 5% immobilized cells of *Saccharomyces cerevisiae* (NCIM 3045) were used for simultaneous alcohol production. The rate of transesterification was low when free cells (5%) of *S. cerevisiae* were used (65% in *Pongamia* and 64 % in *Jatropha*). Maximum ethyl ester production was observed at 36h. Optimum temperature for ethyl ester production was 30°C.

5.1. Introduction:

Transesterification is the process by which triglycerids of fats and oil get converted in to their respective alkyl esters by reacting with primary alcohols. Alkyl esters of fatty acids can be served as a good alternative for mineral diesel. Extensive work is going on production of such fatty acid alkyl esters in the whole world.

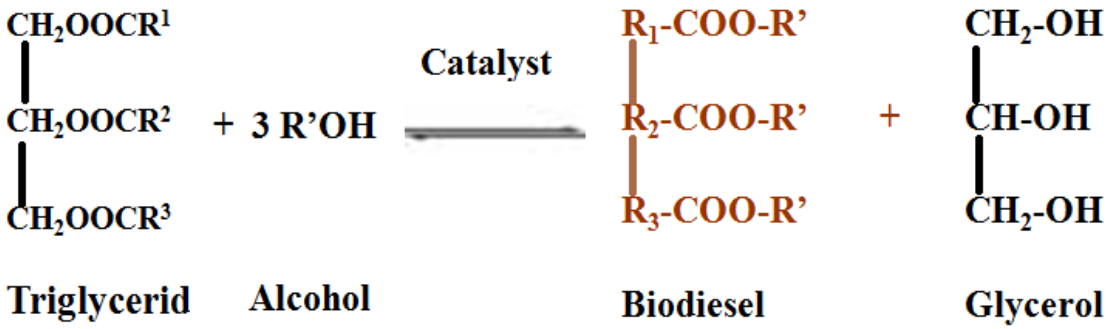


Figure 5.1 Transesterification of triglyceride with alcohol and catalyst.

As shown in the **Figure 5.1**, transesterification reaction is a reversible reaction, excess alcohol is used to shift the equilibrium to the products side. Among the alcohols that can be used in the transesterification process are methanol, ethanol, propanol, butanol and amyl alcohol. Methanol and ethanol are used most frequently. The stoichiometry of the transesterification reaction requires 3 mol of alcohol per mole of triglyceride to yield 3 mol of fatty esters and 1 mol of glycerol **(Figure 5.1).** Higher molar ratios result in greater ester conversion in a shorter time (Fukuda, et. al., 2001).

An important variable affecting the ester yield is the molar ratio of alcohol to vegetable oil. From literature survey it was found that the alcohol required for complete conversion of oil in to alkyl esters is in very high amount. In the transesterification of peanut oil with ethanol, a 1:6 molar ratio liberated significantly more glycerol than a ratio of 1:3 [Feuge and Grose, 1949]. Freedman, *et. al.*, in 1984 studied first time the effect of molar ratios (from 1: 1 to 1:6) on ester conversion with vegetable oils. Soybean, sunflower, peanut and cotton seed oils behaved similarly, with the highest conversion being achieved at a 1:6 molar ratio of oil to alcohol. Krisnangkura and Simamaharnnop in 1992, transesterified palm oil at 70°C in an organic solvent with sodium methoxide as a catalyst and found that the conversion increased with increasing molar ratios of methanol to palm oil. When a large amount of free fatty acids are present in the oil, a

molar ratio of oil to alcohol as high as 1:15 is needed under acid catalysis [Sprules and Price, 1950]. Tanaka, et. al., in 1981 reported two-step transesterification of oils and fats such as tallow, coconut oil and palm oil, used 1:6 to 1:30 molar ratios of oil to alcohol with alkali catalysis to achieve a conversion of 99.5%.

The high amount of alcohol required for alcoholysis of oils will hampers the reaction rates in case of enzymes when used as catalyst. As an alternative to this a system of step wise addition of alcohol was developed for immobilized *C. anturctica* lipase (Novozym 435) which is the most effective for methanolysis among lipases tested [Shimada, et. al., 1999]. Since the enzyme was inactivated by shaking in a mixture containing more than 1:1.5 molar equivalents of oil against the methanol, stepwise addition of methanol was done to avoid lipase inactivation. The excess amount of methanol remained as droplets dispersed in the oil. Lipase may get inactivated when it comes in contacts with these methanol droplets [Shimada, et. al., 1999]. Similar kind of studies have been carried out by using the whole cells as catalysts to achieve the maximum conversion. Ban et. al., in 2001 first time demonstrated efficient catalysis of vegetable oil with the help of biomass support particles (BSPs) of *R. oryzae* with methanol. Stepwise addition of methanol was recommended to minimize the negative effect of methanol on the activity of *R. oryzae* whole cell. However, stability of the whole cell during repeated uses was poor when methanol was used as an acyl acceptor.

The two problems of requirement of high amount of alcohol for complete transesterification and inactivation of lipase due to excess amount of ethanol or methanol are addressed in this **Chapter**. In present study simultaneous production of ethanol was achieved using immobilized *Saccharomyces cerevisiae* during transesterification.

5.2. Materials and Methods

5.2.1. Materials

Sodium alginate, Oleic acid methyl ester, Fatty acid methyl ester mix and heptadecanoic acid methyl ester (served as the internal standard) were purchased from Sigma and were chromatographically pure. Corn steep liquor (CSL) was gift from Hindustan Antibiotics Ltd. Pune, India. *Jatropha* and *Pongamia* oil were purchased from local market. All other chemicals were obtained commercially and were of analytical grade.

5.2.2. Microorganisms

Rhizopus strain *JK 1* was maintained on MGYP (Malt Extract- 0.3%, Yeast Extract- 0.3%, Peptone- 0.5% and Glucose-2%) medium slants stored at 4°C and subcultured every 15-30 days. 10 ml sterile medium was inoculated with spores (approximately 10^6/ml) from fresh agar slant culture and incubated for 48 hr at 30°C and 180 rpm. This seed culture was used throughout the studies. *Saccharomyces cerevisiae (*NCIM 3045) was adapted to high sugar concentration with high efficiency of alcohol production.

5.2.3. Methods

5.2.3.1. *Cells cultivation*

Rhizopus JK-1 was grown under optimum culture conditions for production of extracellular lipase as described earlier [Kantak, et. al., 2011and 2012]. The organism was cultivated in 250 ml Erlenmeyer flasks containing 25 ml of double strength basal medium (Glucose 2%, Na_2NO_3 0.2%, MgSO4 0.1, KH2PO4 0.2%, CSF 6%) seeded with 48h grown seed culture prepared as described earlier. Flasks were incubated on a rotary incubator for 72 h at 30 °C and 180 rpm.

5.2.3.2. Immobilization of *Saccharomyces cerevisiae [NCIM 3045]*

Saccharomyces cerevisiae cells were grown in MGYP medium for 48h at 28°C. Cells were harvested by centrifugation at 7000 rpm for 20min and washed with sterile distilled water. These cells were stored at 4°C and used for immobilization. Five gram cells (wet weight) were immobilized using 2% sodium alginate. Beads were prepared in the ice cold calcium chloride (2%) solution by ionotropic gelation. Entrapped cells were cross-linked to avoid leaching of the cells with 0.05% glutraldihyde for 4h at 4°C. Beads were allowed to hardened again in calcium chloride solution for overnight. The profile of these beads was checked for alcohol production with two different sugars and simultaneous alcohol production and transesterification sucrose and glucose respectively.

5.2.3.3 Studies on alcohol production by immobilized *Saccharomyces cerevisiae:*

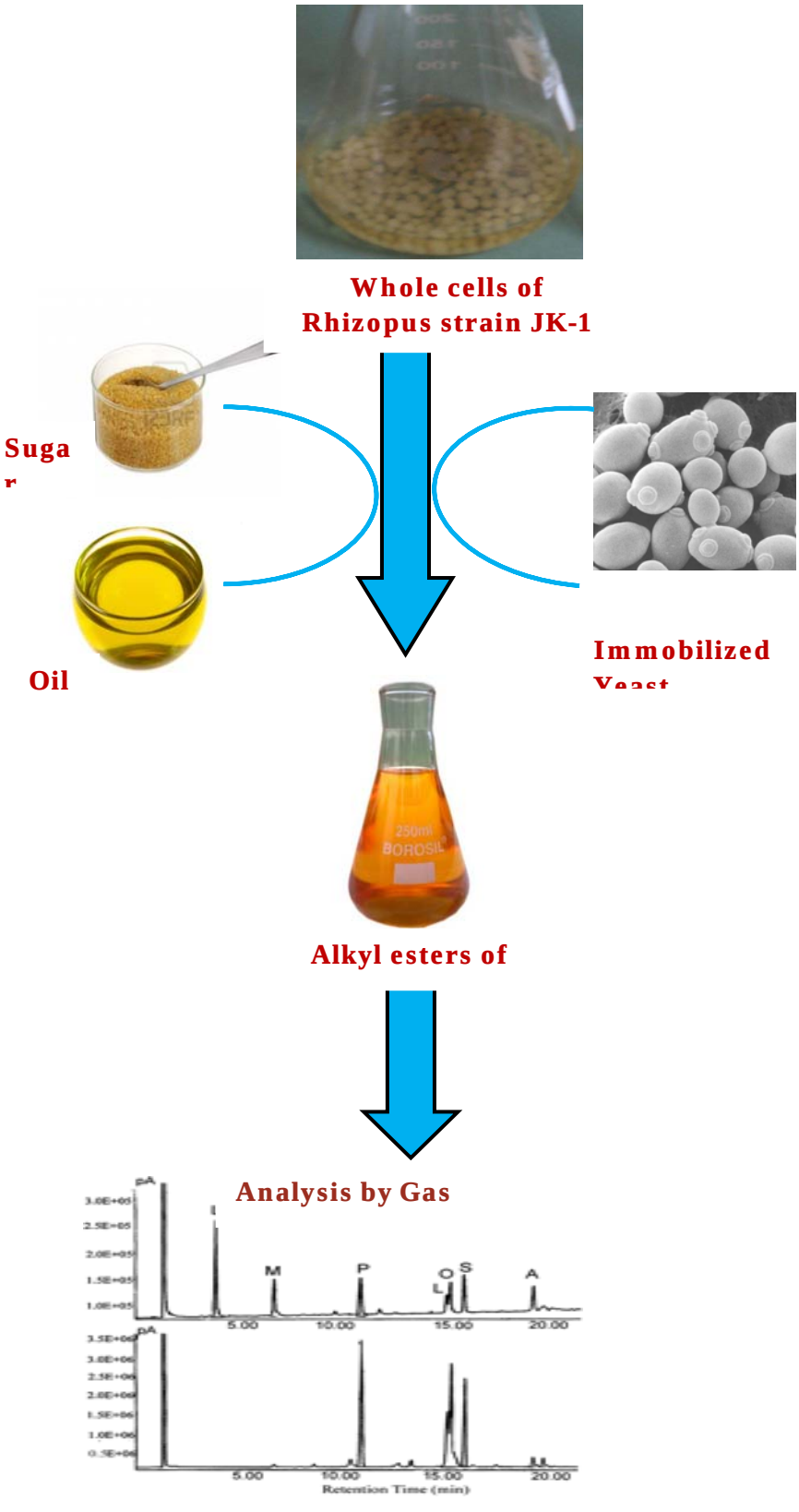
To study the pattern of alcohol production by immobilized *Saccharomyces cerevisiae* glucose and sucrose fermentation was carried out at 30°C up to 72h. Glucose and sucrose concentration studied was from 10-40%. The maximum concentration which gave maximum conversion of glucose and sucrose to ethanol was used in rest of the studies.

5.2.3.4. Effect of cell loading on alcohol production

To study the effect of cell loading on alcohol production, different cell loading ranging from 5-15% wet wt. were immobilized in the 2% sodium alginate as described in the section 5.2.2.2. Fermentation was carried out at 30°C up to 72h using two different sugars, glucose and sucrose. Sugar concentrations used were ranging from 5-30% w/v.

5.2.3.5. Simultaneous alcohol production and Transesterification:

The transesterification reactions were carried out in 250ml Erlenmeyer flasks containing 72h grown extracellular lipase producing *R. oryzae* whole cells in 25ml of double strength basal medium as mentioned in **Chapter IV part B.** To the same flask 10g immobilized 5% (w/v) cells of *Saccharomyces cerevisiae* and glucose was added. Immobilized cells (with 5 %cell loading) were introduced to fermentation medium after completion of 72h growth of Rhizopus strain JK-1 in 250ml flasks. Beads were packed in nylon bag which has pore size big enough to allow easy availability of sugar. Care was taken to choose the mesh size of nylon bag that beads do not escape out in the medium. Transesterification reaction was started after 72h incubation by adding oil (2g) to these flasks. Flasks were incubated at 30°C and 100 rpm. After adding oil rpm was low so as to keep conditions microaerophilic for alcohol production. For the first feed of glucose 10ml of 70 % glucose or sucrose solution was added at the time of initiation of transesterification reaction after 24h of incubation second feed of 10ml of 70% glucose/sucrose was given to *Saccharomyces cerevisiae*. Sugar was added in such a concentration so that it should not exceed over effective concentration of 30%. This two steps addition of glucose/sucrose was done to avoid the production of ethanol in high concentration before 24h and making ethanol available step wise. Two millilitres of samples were withdrawn from the reaction mixture after every 12h time interval, centrifuged to obtain the upper layer and diluted with n-hexane, analyzed by capillary gas chromatography.



Schematic Diagram for Simultaneous alcohol production and transesterification

5.2.3.6. Gas chromatography (GC) analysis

Ethyl esters formed in the reaction mixture were analysed by Gas Chromatograph with Flame –ionization detector (Master GC DANI, Italy). The capillary column (HP-INNOWax, Agilent Technology, USA) used had a length of 30 m with a internal diameter of 0.25mm. Nitrogen was used as the carrier gas at a constant flow rate of 4 kg cm^{-2}. The column oven temperature was programmed from 80 to 210°C (at the rate of 50 °C/m) and hold at this temperature for 6m. The oven temperature is further increased to 230°C (at the rate of 5 °C/ m) with same temperature held for 8m. The injector and detector temperatures were 230 and 240 °C respectively.

5.2.3.7. Effect of *Saccharomyces cerevisiae* cell loading on simultaneous alcohol production and transesterification

To study the effect of *Saccharomyces cerevisiae* cell loading on the simultaneous alcohol production and transesterification, immobilized beads were prepared using different cell loading such as 5 % , 10 % and 15% using 2% sodium alginate and used for alcohol production required for transesterification reaction. The best cell concentration which gave maximum ethyl alcohol was used for further studies.

5.2.3.8. Transesterification using free and immobilized *Saccharomyces cerevisiae*

To study simultaneous alcohol production and transesterification, both free and immobilized cells of *Saccharomyces cerevisiae* were used. Five percent free cells were used for simultaneous alcohol production and transesterification. Similarly immobilized system with 5 % w/w cell loading was used for transesterification. Rest of the transesterification was same as described earlier including time of addition of sugar and oil. Sampling was done after each 12h and analysis was done by Gas Chromatography.

5.2.3.9. Effect of time on simultaneous alcohol production and transesterification

Progress of simultaneous alcohol production and transesterification reaction was monitored up to 48h at 30°C with 100 rpm. Transesterification reaction was carried out essentially as stated in section *5.2.2.4.* Samples (2ml) were collected after every 12h interval. Samples were centrifuged and upper layer was diluted in n-hexane. Analysis was done by Gas Chromatography as described above.

5.2.3.10. Effect of Temperature on simultaneous alcohol production and transesterification

To study the influence of temperature on simultaneous alcohol production and transesterification, reaction was carried out at different temperatures ranging from 25-45°C. After 72h growth of *Rhizopus* strain JK-1 at 30°C at 180rpm, immobilized *S. cerevisiae*, glucose and oil were added as described earlier, flasks were incubated for 48h in rotary shaker with 100 rpm at respective temperatures. Samples (2ml) were aliquoted after each 12h interval, centrifuged and upper layer was diluted with n-hexane. Analysis was done by Gas Chromatography as described earlier in section *5.2.3.6*.

5.2.3.11. Repeated use of immobilized *Saccharomyces ceriviceae*

After every cycle of transesterification immobilized beads were removed by removing the beads containing nylon wire bag. This was done for ease of handling and reuses. Beads were washed thoroughly with sterile distilled water, till they were free of substrate and products. Beads were stored in distilled water at 10°C overnight till used further. Every new cycle was initiated as described earlier with freshly grown *Rhizopus* strain

JK-1 culture. Sampling and analysis of samples from each cycle was done as mentioned above.

5.3. Results and Discussion:

The concept of simultaneous alcohol production and transesterification was introduced first time in this study with maximum production of *Pongamia* and *Jatropha* oil ethyl esters up to 94.05-95%. After introducing such a new concept, problems associated with alcohol requirement and step wise addition of alcohol can be solved effectively. For simultaneous alcohol production both free and immobilized cells of *Saccharomyces cerevisiae* were studied. Two very important parameters which affect the optimum production of ethyl esters like time and temperature were also studied. Maximum ethyl esters were produced after 36h of reaction at 30°C. Immobilized cells of *Saccharomyces cerevisiae* were reused up to six cycles without loss of activity.

5.3.1. Studies on alcohol production by immobilized *Saccharomyces cerevisiae*

Yield of alcohol using immobilized *Saccharomyces cerevisiae* with 5 % cell loading was 13.56 g % from 30 g % of glucose after completion of 24h under above mentioned fermentation conditions. As described in **Chapter IV b** maximum ethyl ester production was observed after completion of 36h of transesterification. Supplementation of sugar was done in two steps to get requisite amount of alcohol required for transesterification and fulfilling step wise addition of alcohol. This helped ses alcohol in to the medium as higher concentration of alcohol leads to inactivation of enzyme and whole cells (Ban, et. al., 2001). Sucrose was also tested for ethyl alcohol production using *Saccharomyces cerevisiae* free as well as immobilized cells. When Sucrose was used in 30 g %

concentration, maximum conversion of ethanol observed was 11.45 % (w/v) after completion of 24h of fermentation.

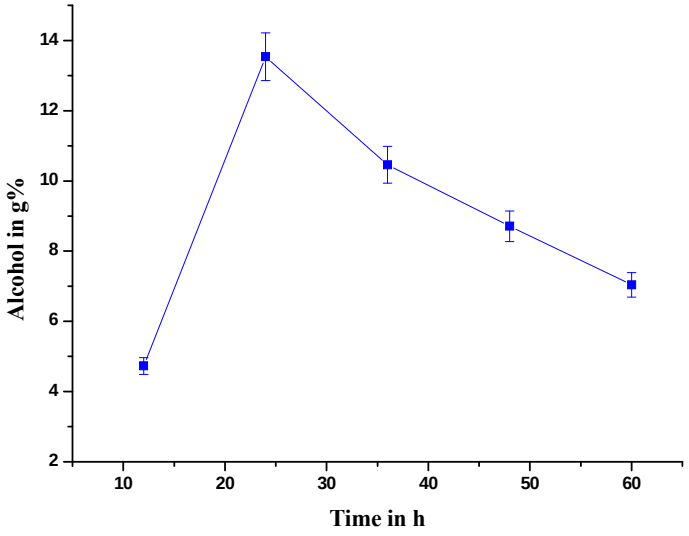


Figure 5.2. Alcohol production by immobilized 5% *Saccharomyces cerevisiae using* glucose as carbon source

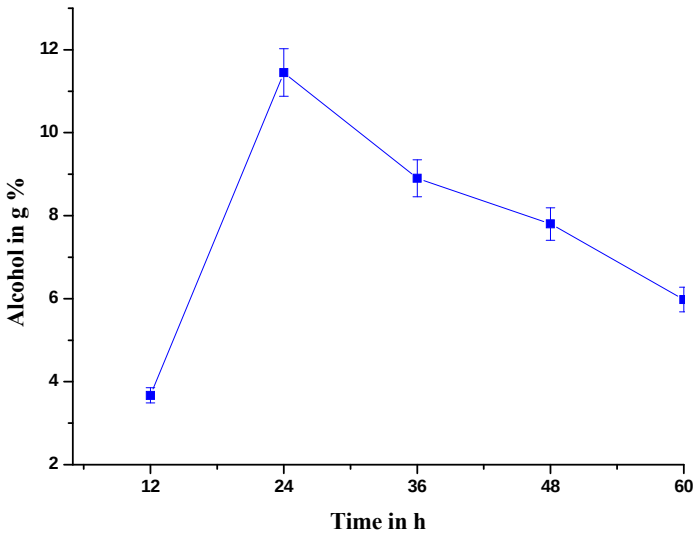


Figure 5.3. Alcohol production by immobilized 5% *Saccharomyces cerevisiae* using sucrose as carbon source.

5.3.2. Transesterification using free and immobilized *Saccharomyces cerevisiae*

Saccharomyces cerevisiae was applied both in free and immobilized form for simultaneous alcohol production eventually transesterification. Maximum conversion of oil into ethyl ester was achieved after 36h when flasks were incubated at 30°C and 100 rpm. Shake flask conditions were adjusted to get required conditions for mass transfer of media contents as it is a co-culture of *Rhizopus* strain JK-1 and *Saccharomyces cerevisiae (NCIM 3045).* The thin layer of oil in fermentation medium maintained the microaerophilic environment required for alcohol production under low shaking conditions. Immobilized cells with 5% w/v cell loading showed promising results than same amount of free cells of *Saccharomyces cerevisiae.* Higher cell loading of immobilized system did not show any significant increase in alcohol fermentation. This can be attributed to low permeability to sugars into immobilized cells which got entrapped in the core of the beads. Same amount of cells when used in the free form showed considerably low transesterification. This effect may be due to low stability of free cells to high alcohol percentage reached after 24h. Immobilized cells (98.50 using glucose and 97.80% using sucrose) showed promising results than free cells (66.01using glucose and 64.09% using sucrose) for the production of *Pongamia* and *Jatropha* oil ethyl esters. Immobilized cells got protected by the matrix hence do not come in direct contact with media environment which contains oil and alcohol.

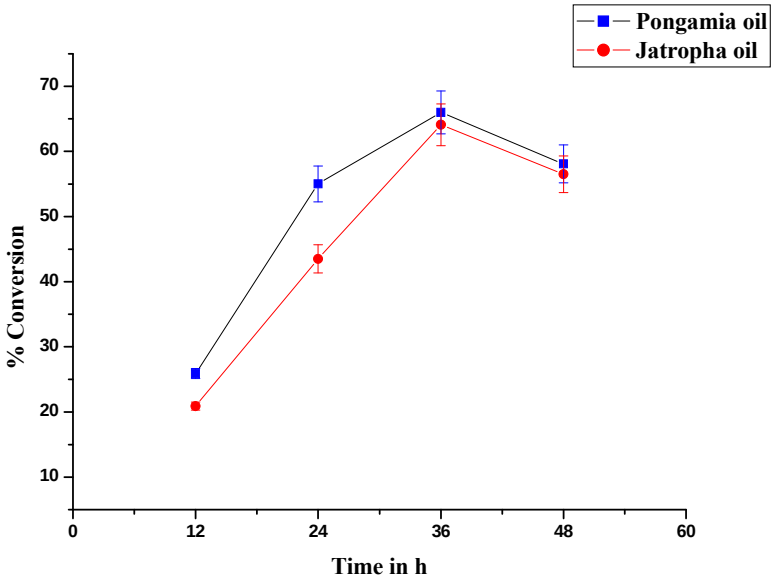


Figure 5.4. Simultaneous alcohol production and transesterification using 5% free cells of *S. cerevisiae* for alcohol production

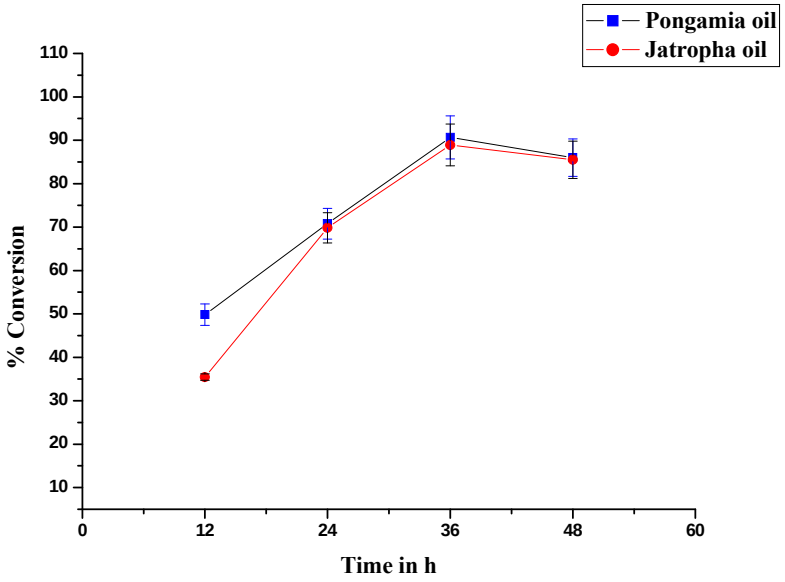


Figure 5.5. Simultaneous alcohol production and transesterification using 5% immobilized cells of *S. cerevisiae* for alcohol production

5.3.3. Effect of time on simultaneous alcohol production and transesterification

Time is very important variable to study for any biochemical reaction. It was observed that time affected the *simultaneous* alcohol production and transesterification. Maximum ethyl ester production was observed at 36h of incubation after addition of oil and immobilized *S. cerevisiae* with requisite amount of either glucose or sucrose. *Pongamia* oil ethyl ester production was slightly higher than that of *Jatropha* oil ethyl esters. In this study we could show effectively that simultaneous alcohol production while transesterification showed optimum conversion efficiency which didn't affect the rate of reaction.

Immobilized cells were administrated to *Rhizopus* cells containing flask after 72h, when extracellular lipase production is maximum. Addition of sugar and yeast cells at this point initiated alcohol fermentation under conditions described in materials and methods. Alcohol produced by fermentation was utilized for transesterification mediated by lipase of *Rhizopus* strain JK-1. As described in previous section 5.3.1 maximum alcohol was obtained after 24h which got reflected in maximum transesterification after 36h. It is essential to add ethanol stepwise to get alcoholysis without hampering the rate of transesterification and for the same stepwise addition of sugar was done to initiate production of alcohol to mimic the stepwise addition of alcohol extraneously.

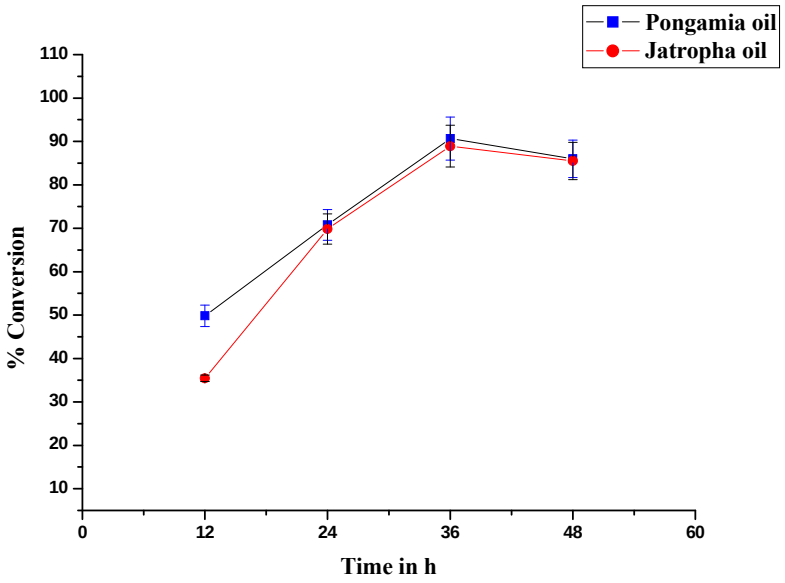


Figure 5.6 Effect of time on simultaneous alcohol production and transesterification by whole cells of Rhizopus strain JK-1.

3.4. Effect of Temperature on simultaneous alcohol production and transesterification

Simultaneous alcohol production and transesterification were carried out at different temperatures ranging from 25-45°C. Maximum transesterification was observed at 30°C. Various lipases and immobilized whole cells were reported to have maximum conversion in the temperature range between 30-35°C. [Ma and Hanna, 1999; Tamalampudi, et. al., 2008; Fukuda, et. al., 2008]. In our previous studies as described in **Chapter II and III** we have shown the optimum temperature for maximum enzyme production was 30°C [Kantak, et. al., 2011 and 2012]. Similar values were obtained when co-culture method was used. This temperature is not only beneficial for fermentation but favourable for simultaneous alcohol fermentation **by *S cerevisiae.***

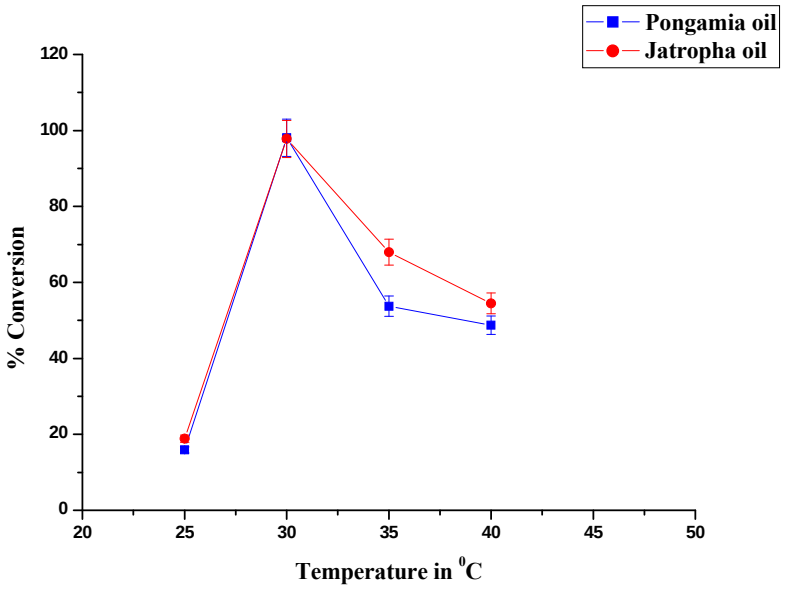


Figure 5.7 Effect of temperature on simultaneous alcohol production and transesterification.

5.3.5. Repeated use of immobilized *Saccharomyces cerevisiae*

After every cycle of transesterification immobilized beads were removed by removing the bead containing nylon wire bag. This was done for ease of handling and reuses. Immobilized *Saccharomyces cerevisiae* cells were used up to six cycles for simultaneous alcohol production and transesterification without much difference in the yield. Reuse of immobilized *S. cerevisiae* cells will be helpful in lowering the cost of ethyl ester production in two ways. Using immobilized cells not only fermented sugar to alcohol but their repeated use made this system for simultaneous alcohol production and transesterification cost effective.

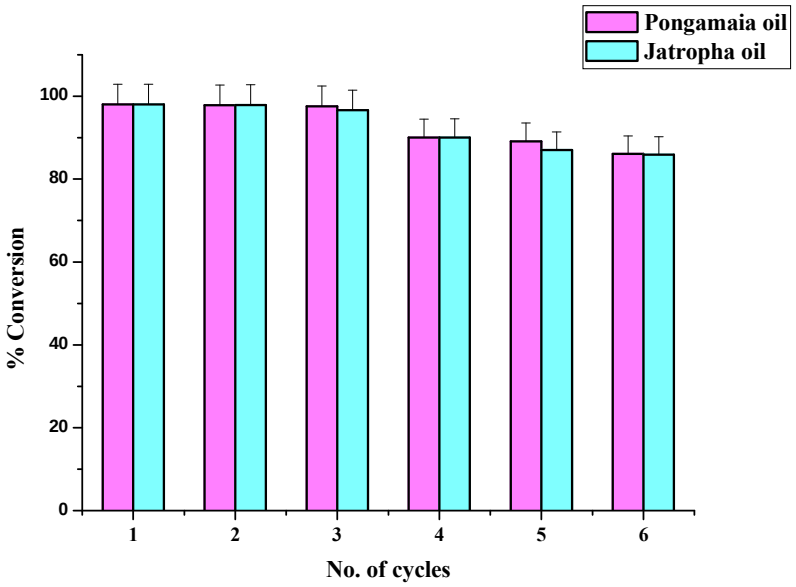


Figure 5.8 Repeated use of immobilized *Saccharomyces cerevisiae* for simultaneous alcohol production and transesterification.

5.4. Conclusion:

A novel method of simultaneous alcohol production and transesterification has been introduced for the first the time. Transesterification of non edible oils was carried out successfully by this process. *S. ceriviceae* can use both glucose and sucrose for alcohol production during transesterification. Different parameters such as time, temperature have been optimized to maximize the transesterification reaction. Uses of immobilized cells of *S. ceriviceae* save the cost of the processes by successful utilization of cells for 6 times.

Chapter VI

Future prospect

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Future scope of the present work

- Lipase is known to have application in various industries. Therefore there is lot of scope in screening and identification of novel lipase producing new microorganisms. Fungal sources for lipase production were having added advantages when compared with the others. Various lipase sources are reported but very less work was carried out in the field of optimization and purification of lipases from novel sources.
- The screening studies conducted by author of the Thesis led to identification of new isolate *Rhizopus strain JK-1* producing extracellular lipase. The culture may give promising productivity by using media manipulation and standardization of cultural conditions. The higher production of lipase from the culture may lead to easy purification of enzyme.
- Attempt towards purification and characterization of enzyme can also be carried out which might be favourable to the industries. Further studies on biochemical and biophysical parameters of the enzyme may provide some additional information about applications of present enzyme in various industries.

- Purified lipases with special feathers like temperature stability are haveing their own importance when used in various industries like detergent and etc. Present lipase is having good temperature stability and can be good candidate for use in such industries.
- Lipases are the enzyme which works in various organic reactions such as transesterification. Considering this fact the purified lipases should be suitable in organic solvents.

- Present lipase was used for the transesterification of non-edible oils such as Pongamia and Jatropha. One more novel source of lipase which can be used for production of biodiesel has been invented. Ethyl ester production rate can be increased by increasing the enzyme concentration and immobilization of enzyme.
- Whole cells of isolate Rhizopus stain JK-1 was used as a biocatalyst for the transesterification of Pongamia and Jatropha oil. The work carried out by the author of the Thesis such as optimization of parameters for maximum ethyl ester production gave maximum conversion of both the oils in to ethyl esters. Scaling up of the process may lead to introduction to a easy process of biodiesel production by using the extracellular lipase producing whole cells.
- A new idea of simultaneous alcohol production and transesterification has been invented by the author of the thesis which can be the big mile stone in biodiesel industry.

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List of Publications

Publications:

- 1.Isolation, Identification and Optimization of a New Extracellular Lipase Producing Strain of Rhizopus sp**. Jayshree B. Kantak** , Aditi V. Bagade, Siddharth A. Mahajan, Shrikant P. Pawar , Yogesh S. Shouche , Asmita Ashutosh Prabhune. Appl Biochem Biotechnol (2011) 164: 969–978

- Characterization of smallest active monomeric lipase from novel *Rhizopus* strain: Application in transesterification. **Jayshree B. Kantak** and Asmita Ashutosh Prabhune (2012) Appl Biochem Biotechnol 166:1769–1780.

- *Rhizopus JK-1* Whole-Cell-Catalyzed Biodiesel Production from Pongamia and Jatropha oil in aqueous system. (Paper Communicated)

- *Simultaneous alcohol fermentation and transesterification of* Pongamia and Jatropha oil *with* Whole cells of *Rhizopus JK-1.* (Paper Communicated)

Posters presented:

- Isolation & identification of industrially important lipase producer *Rhizopus* strain JK-1*:* partial purification and characterization of enzyme. (Accepted in Biotech Research Socity, India, 2010 at Madurai Kamraj University).