LIPASE CATALYZED TRANSESTERIFICATION REACTIONS OF TRIGLYCERIDES

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by

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Madhu Katiyar

Madhu Katiyar

CERTIFICATE

This is to certify that thesis entitled "LIPASE CATALYSED TRANSESTERIFICATION REACTIONS OF TRIGLYCERIDES", being submitted by Madhu Katiyar, to the School of Chemistry and Biochemistry, Thapar University, Patiala for the award of degree of DOCTOR OF PHILOSOPHY, is a record of bonafide research work carried out by her. Ms. Madhu Katiyar has worked under my guidance and supervision and has fulfilled the requirements for the submission of this thesis, which to my knowledge has reached the requisite standard.

The results embodied in the thesis have not been submitted in part or full to any other University or Institute for the award of any degree or diploma.

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CONTENTS

List of Abbreviations

Abbreviation	Description
BET	Brunauer- Emmett-Teller
cm	centimetre
EDX	Energy Dispersive X-ray
FAMEs	Fatty acid methyl esters
FAEEs	Fatty acid ethyl esters
FESEM	Field emission scanning electron microscope
FFAs	Free fatty acids
FT-IR	Fourier transform infra red
FT-NMR	Fourier transform nuclear magnetic resonance
GC-MS	Gas chromatography mass spectrometry
XRD	X-ray diffraction
g/mmol	Gram per millimole
µg/ml	Micro gram per millilitre
IU	International unit
kcal mol^{-1}	Kilo calorie per mole
kJ mol^{-1}	Kilo joule per mole
L $mol^{-1}cm^{-1}$	litre per mole centimetre
MeOH	Methanol
EtOH	Ethanol
min	minute
mL	Millilitre
MO	Methyloleate
MHz	Mega hertz
Mol	Mole
mg	Milligram
mg L^{-1}	Milligram per litre
µmol	Micro mole
N_2	Nitrogen
nm	nanometre
ppm	Parts per million
pI	isoelectric point
rpm	Rotation per minute
TLC	Thin layer chromatography
TEM	Transmission electron microscope
TOF	Turn over frequency
UV-vis	Ultra violet-visible
U/g	Unit per gram
v/v	Volume by volume
wt%	Weight percentage

List of Symbols

Symbols	Description
Å	Angstrom
A	Pre–exponential factor
C	Celsius
E_a	Activation energy
g	Gram
h	Hour
K	Kelvin
k	Rate constant
L	litre
M	Molar
m	Metre
%	percentage
μ	Micro
θ	Theta
α	Alpha
°	Degree
R_f	Retention factor
R	Gas constant
T	Temperature
t	Time
v	Volume

List of Figures

List of Tables

Abstract

Fatty acid alkyl esters (FAAEs), commonly known as biodiesel, has emerged as environmental friendly and renewable substitute for the conventional diesel fuel, in recent past. Biodiesel at commercial scale is produced by the transesterification of triglycerides (animal fats or vegetable oils) in presence of chemical catalysts. Chemical catalysts due to their inherent disadvantages must be replaced with greener and renewable catalysts such as lipase. On the other hand slow rate of lipase catalyzed reactions and extremely high cost of pure enzyme has prohibited their application at industrial scale for biodiesel production.

In this context, present work envisaged to enhance the activity and reusability of the of the commercially available lipases. The effect of alkali (Na^{+} and K^{+}), alkaline earth (Ca^{+2} and Ba^{+2}) and transition (Cr^{+3}, Fe^{+3}, Co^{+2}, Cu^{+2} and Ni^{+2}) metal ions on hydrolytic and transesterification activity of *Candida rugosa* lipase (CRL) and commercially available immobilized lipases (Lipozyme and Novozyme 435) was investigated. Out of the metal ions studied, Cr^{+3} and Co^{+2} were able to catalyze the activity of the pure lipase to the maximum extent. However, activity of the immobilized enzymes was not increased by the presence of metal ions. The kinetic of the lipase catalyzed reactions in presence of metal ions were also investigated.

To improve the reusability and stability, lipase enzyme was immobilized by physical adsorption and entrapment techniques using mesoporous silica as a support material. Mainly three different types of support materials *viz.,* MCM-41, SBA-15 and Ti/SiO_2, were employed for the physical adsorption of lipase. The immobilized lipase has been characterized by powder X-ray diffraction, scanning and transmission electron microscopic and Fourier transform infrared studies. The immobilized enzyme was used as solid biocatalyst for the transesterification reactions of cotton seed oil with methanol. Physically adsorbed demonstrated poor reusability may be due to the denaturation of the enzyme while catalyzing the first catalytic cycle.

On the other hand, *Rhizomucor miehei* lipase was entrapped in a single step with in silica particles having oleic acid core. Immobilized lipase was characterized by powder X-ray diffraction, scanning and transmission electron microscopy and Fourier transform infra-red studies. The immobilized enzyme was employed for the transesterification of the cotton seed oil with methanol and ethanol. Under the optimum reaction condition of methanol to oil molar ratio of 12:1, stirring speed of 250 rpm at 40 ºC, and biocatalyst concentration of 5

wt% (with respect to oil), complete transesterification (> 98% fatty acid methyl ester yield) was achieved in 16 h of reaction duration. The enzyme was amenable to recovery and reuse for the twelve successive catalytic cycles. Moreover, the entrapped enzyme didn't demand low temperature storage condition and found to be stable and active even after eight months of storage at room temperature (25-30°C). The activation energy for the immobilized enzyme catalyzed ethanolysis and methanolysis were found to be 8.36 and 4.72 kcal mol^{-1}, respectively.

Keywords: Immobilization, Transesterification, Biodiesel, Powder X-ray diffraction, Scanning electron microscopy, Transmission electron microscopy, Fourier transform infra red spectroscopy, and Kinetics.

Chapter-1

Introduction and literature review

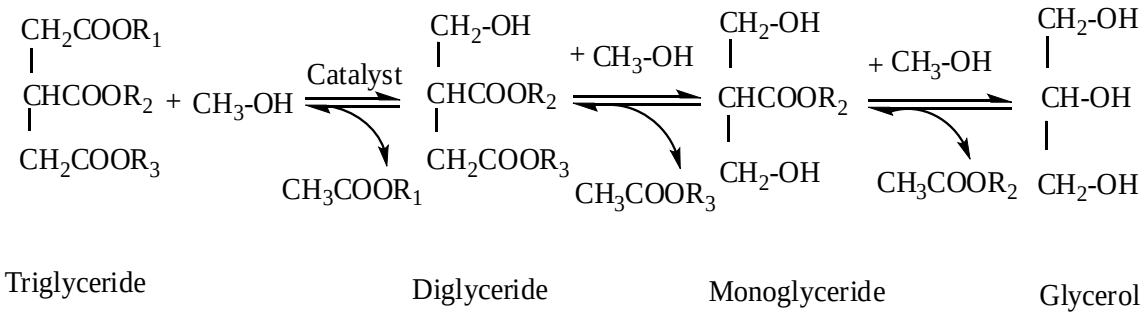
Abstract: Fatty acid alkyl esters (FAAEs), commonly known as biodiesel, has emerged as environmental friendly and renewable substitute for the conventional diesel fuel, in recent past. Biodiesel at commercial scale is produced by the transesterification of triglycerides (animal fats or vegetable oils) in presence of chemical catalysts. Chemical catalysts due to their inherent disadvantages must be replaced with greener and renewable catalysts such as lipase. Pure lipase is extremely expensive and hence, must be immobilized over a solid support to prepare reusable solid biocatalyst. Physical adsorption of lipase over a support is frequently used technique for the lipase immobilization, although it suffers the problem of enzyme leaching in the reaction mixture. Covalent bonding of the lipase with the functional groups anchored on matrix surface, cross linking and entrapment are other techniques reported for the lipase immobilization in order to make the solid biocatalyst more stable and reusable.

Keywords: lipase immobilization, transesterification, reusability, physical and chemical immobilization

1.1 Introduction

Economic development of many developing countries and increasing population has lead to huge increase in the energy demand. Environmental concerns, depleting source of fossil fuel and escalating crude oil price demand the replacement of conventional fossil fuel with renewable one such as biodiesel. Biodiesel, chemically known as fatty acid methyl esters (FAMEs), has emerged as an ecofriendly substitute for the conventional diesel fuel in recent past. It has several advantages over conventional diesel fuel, such as, it is derived from renewable resources (Tang *et al.,* 2013), free from aromatic compounds, causes lower emissions of carbon monoxide, particulate matters, sulphur compounds and greenhouse gases due to its closed carbon dioxide cycle (Reddy *et al.,* 2010; Kulkarni *et al.,* 2006; Madankar *et al.,* 2013). At industrial scale, biodiesel is produced by chemically catalyzed transesterification (also known as alcoholysis) of triglycerides (vegetable oils and animal fat) with methanol (Yucel *et al.,* 2011; Tang *et al.,* 2013; Madankar *et al.,* 2013). Glycerol, a valuable molecule for the pharma and skin care industry, is a by product of this reaction (Pradhan *et al.,* 2012; Aybastier and Demir, 2010).

Transesterification of a triglyceride molecule with alcohol completes in a series of three successive reversible reactions (Dossat *et al.,* 2002). At every step, an ester molecule is produced besides the formation of diglyceride, monoglyceride, and glycerin in first, second and third step, respectively as shown in Scheme 1.1.



Scheme 1.1. Three successive steps of transesterification reactions of triglycerides.

At commercial scale, transesterification reaction is usually carried out in presence of homogeneous acid (*e.g.*, H_2SO_4, HCl, etc.,) or alkali catalysts (*e.g.*, NaOH, KOH, etc.). Use of chemical catalysts is advantageous as they are easily available, cost effective and generate high yield in short reaction duration (Al-Zuhair, 2005; Kumar and Ali, 2010; Kumar and Ali, 2012; Kaur and Ali, 2011). However, they are not eco-friendly, lead to the formation of catalyst contaminated biodiesel and hence, required washing of the products which generate huge amount of the industrial effluents (Vidya and Chadha, 2010; Demirbas and Karslioglu, 2007; Al-Zuhair *et al.,* 2007). Further, alkaline catalysts get deactivated if moisture and free fatty acid (FFA) contents in the feedstock is > 0.1 and 0.3 wt%, respectively.

The problems associated with the application of chemical catalysts could be avoided by replacing them with biocatalysts (enzymes). Lipases (E.C. 3.1.1.3) are ubiquitous and among the most widely used enzymes for the chemical transformations. They recognize a wide variety of the molecules and can catalyze the different reactions such as ester hydrolysis, esterification, transesterification (alcoholysis), interesterification, aminolysis, peroxidation, and epoxidation (Chatterjee *et al.,* 2009; Gupta *et al*., 2013; Yiu and Wright, 2005). Lipase catalyzes the transesterification reaction under ambient conditions and hence the energy demand for the biodiesel production may reduce dramatically (Chatterjee *et al.,* 2009; Talukder *et al.,* 2013). Additionally lipase did not get deactivated *via* saponofication,

which is a common problem associated with the use of chemical catalysts (Dizge *et al.,* 2009a; Tan *et al.,* 2010). However, slow reaction rate of lipase catalyzed reaction and extremely high cost of pure lipase, has prohibited its application at industrial scale (Muniyappa *et al.,* 1996, Cao, 2005; Salis *et al.,* 2008; Zhao *et al.,* 2013; Karimpil *et al.,* 2012). Thus in order to make the lipase cost competitive with chemical catalyst, there is a need to find out to the ways not only to enhance the rate of lipase catalyzed reaction also its reusability.

1.2 Sources of lipase

Lipases are ubiquitous in nature and can be produced by various plants, animals and micro-organisms (Hasan *et al.,* 2010; Villeneuve *et al.,* 2000; Zhang *et al.,* 2012). For industrial applications enzyme is preferably produced from microorganisms because of their shortest generation time (Ribeiro *et al.,* 2011). The other advantages of microorganisms includes high yield of the product from substrate, great adaptability to environmental conditions and, simplicity in genetic manipulation and cultivation conditions (Ribeiro *et al.,* 2011). Moreover, in few cases enzyme producing microorganism may be directly employed for the reaction and, hence prior enzyme isolation and purification is not necessary. Although lipases from different sources are able to catalyze the transesterification reaction, bacterial and fungal lipases usually obtained from *Aspergillus niger, Candida antarctica, Candida rugosa, Chromobacterium Viscosum, Mucor miehei, Pseudomonas cepacia, Pseudomonas fluorescens, Photobacterium lipolyticum, Rhizomucor miehei, Rhizopus oryzae, Streptomyces sp., and Thermomyces lanuginose* (Yahya *et al.,* 1998; Cho *et al.,* 2012), are used in biodiesel formation. Novozyme 435 (*Candida antarctica* lipase immobilized on acrylic resin) and Lipozyme (*Rhizomucor miehei* lipase immobilized on macroporous ion-exchange resin) are the commercially available lipases and extensively used in literature for the transesterification reactions (Xu *et al.,* 2003).

1.3 Lipase structure

The structure of the lipases differ significantly in size and in amino acid sequences with the exception of a pentapeptide, Gly/Ala-X-Ser-X-Gly, motif. However, they are all serine esterases belonging to the α/β hydrolase superfamily containing the N- and C-terminal domain, and amino acids network of α-helices as well as β-sheets (Jaeger *et al.,*

1999; Gupta *et al*., 2013). The molecular weight of the lipase family usually varies in the range of 20-60 kDa. The active site, so called catalytic triad, is composed of serine, histidine and aspartatic acid or glutamic acid residues. The residues of a catalytic triad can be far from each other in the primary structure amino-acid sequence, however, they are brought close together when the chain folds into its 3-D tertiary structure. A distinctive structural feature, common to most lipases, is the presence of a lid or flap to cover the lipase active site. This lid is composed of an amphiphilic α-helix peptide sequence and in closed conformation (*i.e.*, in the absence of an interphase or organic solvent) it prevents the access of substrate molecule to the catalytic triad. When lid is opened a large hydrophobic surface is created to which substrates (usually triglycerdides) can bind. This presumed mechanism is supported by the X-ray structures of lipases covalently complexed with hydrophobic inhibitors such as alkyl phosphonates, cycloalkyl phosphonates, or alkyl sulfonates (Polgar, 2005; Bhushan and Tanwar, 2009).

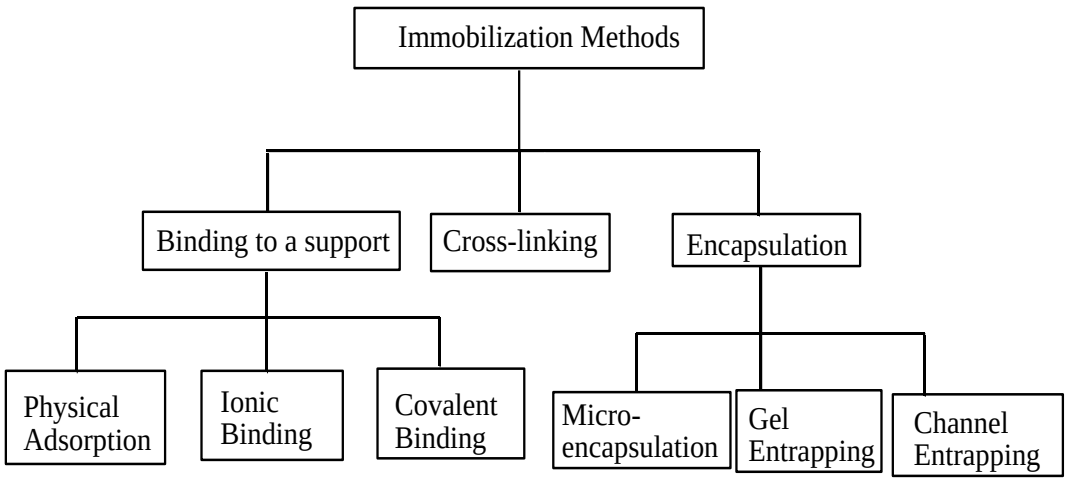
1.4 Applications of lipase

Lipases are important from application point of view as they have versatile applications and easy to produce at mass scale. They are mainly used in the processing of oil and fats, food processing, leather textile, detergents and degreasing formulations, production of fine chemicals, pharmaceuticals and cosmetics (Freitas *et al.,* 2009; Dizge *et al.,* 2009a). The use of lipases in the oleochemistry is enormous as their uses saves energy and minimizes thermal degradation during hydrolysis, esterification, alcoholysis and aminolysis reactions (Yigitoglu and Temocin, 2010; Kim *et al.,* 2013; Yiu and Wright, 2005).

1.5 Immobilization of lipase

As discussed earlier the major hurdle in the commercial application of lipase is their extremely high production cost. Moreover, enzymes are liable in nature and hence, they may undergo denaturation and diminished activity (Gupta *et al*., 2013). Therefore to prepare the solid and reusable biocatalyst, immobilization of the lipase is necessary. A variety of carriers have been employed for the lipase immobilization, *viz.,* mesoporous silica, celite, polymers, cotton membrane, and amine group functionalized magnetic nanoparticles (Kumari *et al.,* 2009; Cao, 2005; Salis *et al.,* 2008; Xu *et al.,* 2004; Karimpil *et al.,* 2011).

Many diverse immobilization techniques (Scheme 1.2) were reported for the lipase immobilization over these supports such as physical adsorption, encapsulation, entrapment, cross linking and chemical bonding with the functional group anchored on carrier (Karimpil *et al.,* 2012; Tan *et al.,* 2010; Ying *et al.,* 1999).



Scheme 1.2. Overview of different strategies for lipase immobilization

Immobilized lipase is expected to have the following advantages over free enzymes: i. Easy separation and reusability, ii. Formation of the non contaminated product(s), iii. Enhanced pH, chemical and thermal stability, iv. Possibility for the development of the continuous reactor, and v. Cost effectiveness

1.5.1 Physical Adsorption

Immobilization by physical adsorption is the easiest and least expensive technique to prepare solid biocatalysts. In this technique weak forces such as Van der Waals, hydrogen bonds, electrostatic forces and hydrophobic interactions are responsible for the lipase immobilization on matrix (Andrade and Hlady, 1986). Among them, electrostatic interactions are relatively stronger, although the interactions are not strong enough to prevent the enzyme leaching (Manyar *et al.,* 2008). In order to increase the electrostatic interactions between matrix and enzyme it is necessary to adjust the pH of medium with respect to the isoelectric point (pI) of enzyme. A variety of carriers such as mesoporous

silica, celite, polymers, cotton membrane, and amino functionalized magnetic nanoparticles have been employed for the lipase immobilization by physical adsorption technique (Tan *et al.*, 2010). Figure.1.1, explain the interactions between enzyme and mesoporous materials during the adsorption process.

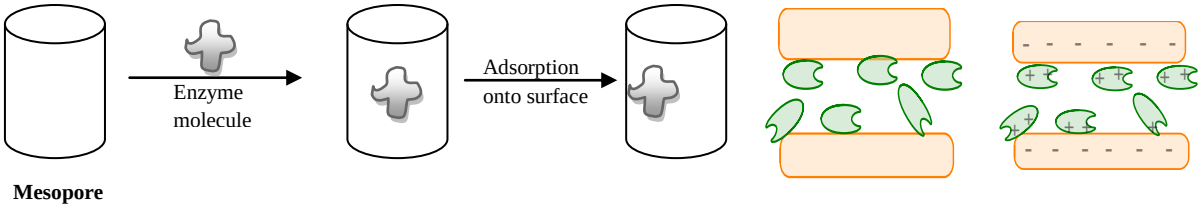


Figure 1.1. (a) Physical adsorption of enzymes in mesoporous materials involving (b) Van der Waals force, hydrogen bonding or hydrophobic interaction (c) electrostatic force of attraction.

In case of mesoporous silica (e.g., SBA15, MSMs, etc.) the interaction could be further enhanced by performing the adsorption at a pH where enzyme is positively charged because silica carries negative charges at pH > 3. The sign of the overall charge on a surface can readily be predicted on the basis of pI (the pH at which the overall charge is zero) and the protein will be positively charged at a pH lower than the pI (Yiu and Wright, 2005). The main drawback of this method, as already discussed, is the poor reusability of the immobilized enzyme.

1.5.2 Covalent bonding

In is evident from the above discussion that physical adsorption technique could not bind the enzyme onto the support firmly and hence, enzyme may leach out during the catalytic run. Covalent binding with the support is another alternative to improve the stability of the immobilized enzyme. Mesoporous materials being non reactive has been proven a better supports for the covalent bonding of enzymes. These materials not only contain high specific surface areas, relative large-pores but also abundant silanol groups which may be further substituted by post synthesis modification with the functional groups

such as alkoxysilanes, chlorosilanes, amine or methallylsilanes as shown in Figure 1.2 (Price *et al.*, 2000; Macquarrie *et al.*, 1999; Stein *et al.*, 2000; Lim and Stein, 1999).

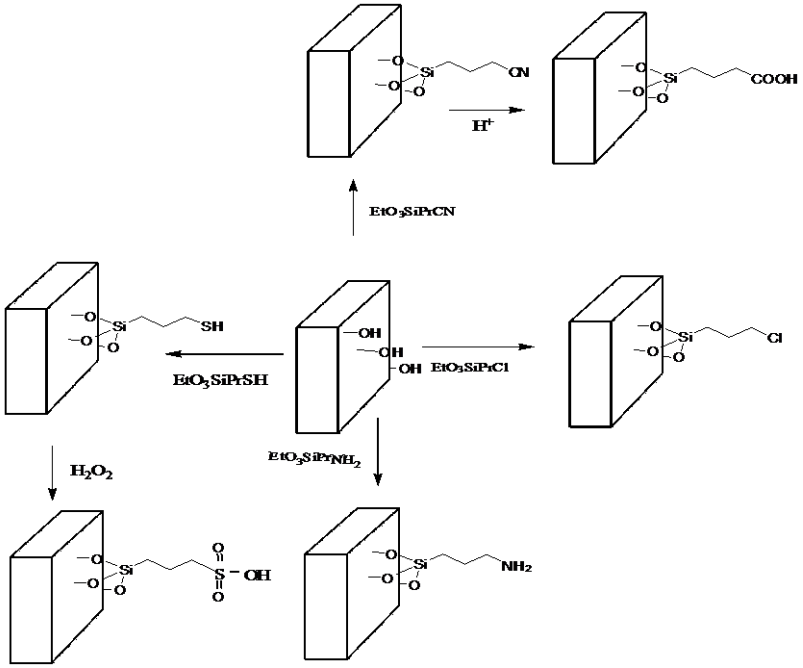


Figure 1.2. Functionallization of mesoporous silica with organic functional group

On the another hand the direct synthesis, called co-condensation, not only allow uniformly distributed high loading of organic groups on the surface of mesoporous material but also preserves high surface area and pore size of the matrix (Li *et al*., 2013). Finally enzyme is covalently immobilized with support by the reaction between surface functional group and -NH_2 (lysine, arginine, and histidine) or -SH (cysteine) residues of enzymes as shown in Figure 1.3.

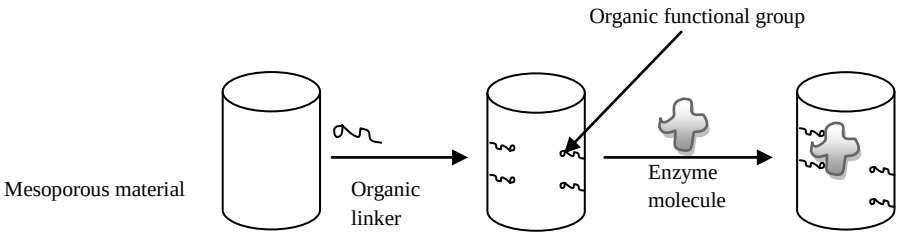


Figure 1.3. Immobilization of enzyme on mesoporous material through covalent bonding

Yucel (2012) reported the T*hermomyces lanuginosus* lipase immobilization *via* covalent bonding on polyglutaraldehyde activated pomace powder. The immobilized lipase was used for the transesterification of the pomace oil and found to be stable even during 10 catalytic runs without significant loss in activity.

The covalently immobilized enzymes were found to be more stable than the soluble lipase and stability of the immobilized enzyme depends upon the chemical/physical nature of the carrier, binding mode and position of the binding (Mendes *et al*., 2011). However, in some cases due the enzyme denaturation, as result of the covalent binding near active site, a significant loss in enzyme activity was observed.

1.5.3 Cross-Linking

In this technique amine groups of enzyme molecules are cross linked together with the help of glutaraldehyde (GA). GA shows many structures in aqueous solution such as monomeric, dimeric, trimeric, polymeric etc. Figure 1.4 shows reaction between aldehyde group of poly-GA and amine group of lipase enzyme which resulted the cross linked lipase aggregates connected together *via* imine (-CH=N-) linkage. The aggregation of enzyme molecules inside the pores of mesoporous materials by physical adsorption followed by their cross linking within the pores by adding GA as cross linking agent has also been reported (Kumari *et al.,* 2007). Now the cross linked enzymes could not leach out from the pores however, substrate molecules still can approach the active sites. It is also possible to prepare the carrier free cross linked enzyme aggregated (CLEAs) of up to 1 μm size. Kim *et al.* (2005), added magnetic nanoparticles to CLEAs immobilized over mesocellular foams (MCFs) or carbon foams to separate them from reaction mixture with the help of magnetic field. Jung *et al.,* (2009) employed different methods for the cross linking of glucose oxidase in MCFs by varying the enzyme to linker ratio and time interval between the addition of enzyme and linker. The resulting biocatalyst was finally used for the oxidation of indole in a fixed bed reactor.

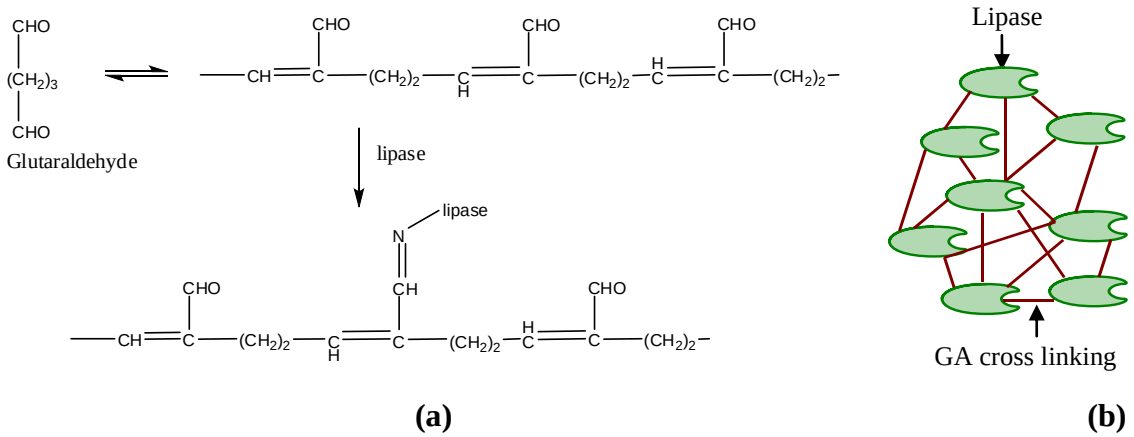


Figure 1.4. Immobilization of lipase by (a) crosslinking with glutaraldehyde (Gupta *et al.*, 2013), (b) a pictorial presentation of the cross linked enzyme.

1.5.4 Encapsulation

To improve the stability of the immobilized enzyme in the cages of the mesoporous material, after adsorption, bulky functional groups (such as 3-aminopropryltriethoxysilane) could be introduced to reduce the pore opening as shown in Figure 1.5a. This procedure allows the enzyme molecules to be trapped within the pores but still permits the reactant and product molecules to diffuse in and out of the pores (Manyar *et al.*, 2008). For example, cytcochrome c was adsorbed in MCM-48 pores followed by silylation of the pore opening to reduce the enzyme leaching (Washmon-Kriel *et al*., 2000, Kim *et al*., 2007). Similarly in case of larger pore size materials such as SBA-15, silylation of the pore aperture with vinyl groups followed by in situ polymerization was employed (He *et al*., 2006). The major disadvantages of these methods are complicated derivatization process of the matrix and risk of the enzyme deactivation during the chemical transformations (Zhou and Hartmann, 2012).

The enzymes can also be encapsulated by enveloping them within semi permeable membranes to form the microcapsules of ~10-100 μm diameters as shown in Figure 1.5b. Enzyme molecules being larger in size cannot come out from capsule, but small substrate and product molecules can pass through the semi permeable membrane (Murty *et al.*, 2002). The main disadvantage of this method is the diffusion problem and advantage is the co-immobilization of several enzymes within the same capsule.

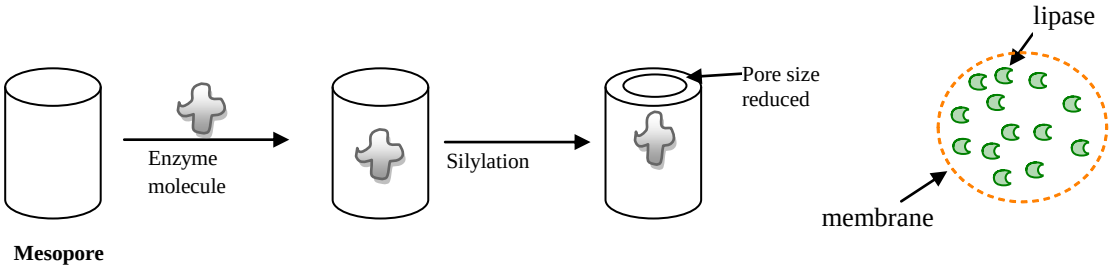


Figure 1.5. Enzyme immobilization by encapsulation method (a) in mesoporous material and (b) within semi permeable membrane.

1.5.5. Entrapment

Entrapment refers to the physical immobilization of an enzyme within the host matrix as shown in Figure 1.6 (Zhou and Hartmann, 2012). In this technique mesoporous silica or polymers are used as matrix to entrap/encapsulate the enzyme molecules. In gel entrapment method lipase is entrapped within the porous support in a single step and no direct bond formation between support material and enzyme molecules is involved, hence, lipase activity remains unaffected upon immobilization. This method is not only simple but also often imparts better reusability, activity and stability to the enzyme towards pH, heat and reactant/product molecules as enzyme remain partially protected by the matrix (Palla *et al.*, 2011; Souza *et al.*, 2012). The method could also be used to entrap even high protein concentration within the support. During the reaction, the substrate is able to penetrate the porous wall of the matrix to reach the enzyme active site but enzyme, due to the small pore size, is not able come out from the matrix.

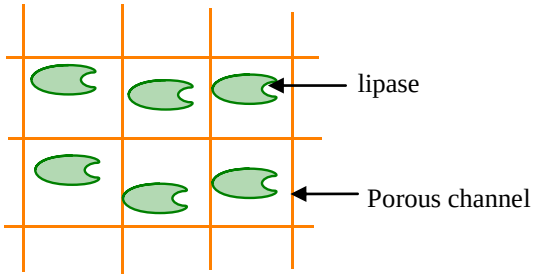
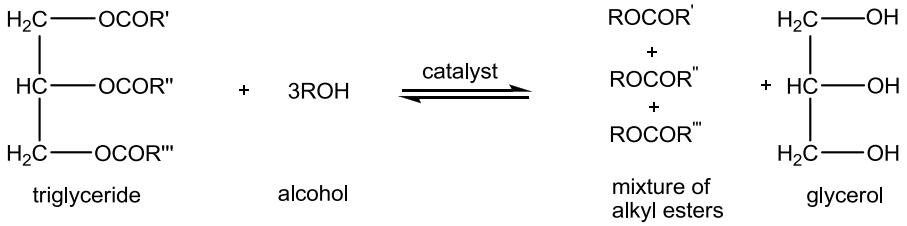


Figure 1.6. Enzyme entrapped within the support materials.

1.6 Application of lipase in transesterification

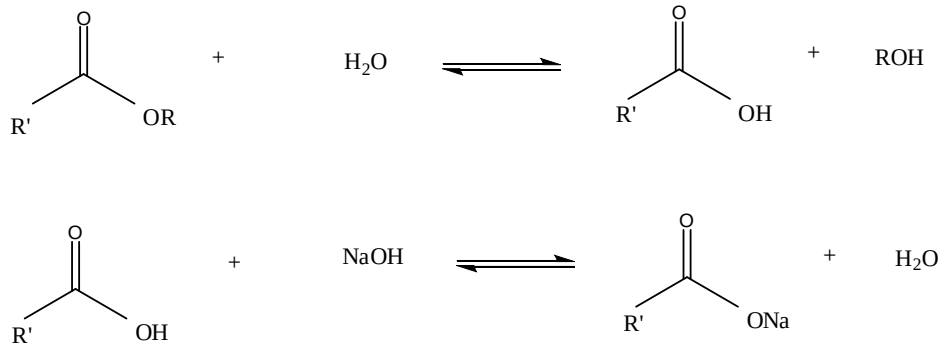
In transesterification reaction one ester group can be interchanged with another one. Transesterification of triglyceride with alcohols leads to the formation of fatty acid alkyl esters, commonly known as biodiesel, and glycerol as shown in Scheme 1.3 (Li and Rudolph, 2008; Vicente *et al.,* 2004).



Scheme 1.3. Transesterification of triglycerides with alcohol to form methyl esters of fatty acids (biodiesel) in the presence of acid, base or enzyme as catalyst. R', R'' and R''' are the hydrocarbon chains of fatty acids, usually having 14-20 carbon atoms.

Further, such type of diesel fuel alternative have economic as well as strategic importance for a country like India, which depend heavily on foreign countries (~70% fossil fuel of total Indian requirement has been imported in last ten years) to meet its demand for fossil fuel (Shukla, 2005).

Most biodiesel at industrial scale is produced by homogeneous alkaline catalysts, such as sodium and potassium methoxides and hydroxides and among them NaOH and KOH are preferred due to their wide availability and low cost. The limitations of such catalysts are the production of alkali metal ion contaminated biodiesel and glycerol, which requires additional purification step. Further, same catalyst required high quality and costly feedstock which must be essentially free from FFAs (< 0.5 wt%) and moisture contents (< 0.1 wt%). High FFA and moisture contents lead to the formation of soap as shown in Scheme 1.4 which, could increase the biodiesel purification and separation cost (Li and Rudolph, 2008).



Scheme 1.4. Hydrolysis of fatty acid alkyl ester in to free fatty acid followed by saponification free fatty acid.

Vegetable oil having high free fatty acid contents (> 0.5 wt %) could be transesterified in the presence of acid catalysts *viz.,* sulphuric, phosphoric, hydrochloric or organic sulfonic acids. The main problems associated with homogeneous acid catalysts included the slow reaction rate, deactivation of the catalyst by the presence of high concentration of moisture, reactor corrosion, and production of the acid contaminated biodiesel and glycerol.

Application of lipase for the transesterification reaction could circumvent the problems associated with the chemical catalysts, as enzyme catalyzed reactions not required energy intensive reaction conditions and also not sensitive towards moisture and FFA contents (Moreira *et al.,* 2007). Lipases isolated from a variety of microorganisms, animals, and plants have been employed for the transesterification of various triglycerides to produce biodiesel (Zhang *et al.*, 2012). The major area of concern related with the lipase application is its extremely high cost in comparison to the chemical catalyst (Cao, 2005; Salis *et al.,* 2008). Use of immobilized enzyme allows the easy physical separation of enzyme from the reaction mixture. The recovered enzyme could be further reused to make the overall process more cost effective (Galarneau *et al.,* 2006).

Kawakami *et al*. (2011) demonstrated the immobilization of *Burkholderia cepia* lipase on hydrophobic silicates and its application towards the transesterification of the jatropha oil to produce biodiesel. The immobilized lipase was used in batch as well as in continuous mode for the transesterificnation of the jatropha oil. Kumari *et al*. (2009) treated the ethanolamine functionalized silica with glutaraldehyde and same support was employed for the immobilization of *Enterobacter aerogenes* lipase by covalent bonding. The

immobilized lipase was found to be efficient towards the transesterification reaction even at 55 °C. Cao *et al*. (1992) demonstrated the transesterification and esterification reaction catalyzed by the *Porcine pancreatic* lipase immobilized on glass and other solid supports. Watanabe *et al*. (2000) used the commercially available immobilized *Candida antartica* lipase (Novozyme 435) for the biodiesel production from vegetable oils. However, only partial conversion was achieved due to the deactivation of enzyme while catalyzing the reaction. Table 1.1 shows different techniques over various support material for lipase immobilization and their application as solid biocatalyst for the transesterification of triglycerides.

Table 1.1. Immobilization of lipase by various techniques and their application for the transesterification of triglycerides

Enzyme source/ Immobilization method	Carrier	Reaction conditions	Maximum FAAE* yield (%) /Reusability	Ref.
		Alcohol to oil (m/m), temp (°C), time (h)		
Physical adsorption				
Candida antartica	Acrylic resin	6:1, 40, 42	80/ nil	Wang *et al.*, 2007
Pseudomonas fluorescens	Macroporous polypropylene	8:1, 30, 22	58 /nil	Salis *et al.*, 2008
Pseudomonas cepacia	Macroporous polypropyl	8:1, 30, 51	37/nil	Salis *et al.*, 2008
Candida rugosa	MCM-41	12:1, 40, 48	98/2	Katiyar and Ali, 2012
Chemical binding				
Burkholderia cepacia	Silica–PVA	7:1, 39, 48	98/nil	Freitas *et al.*, 2009
Candida antarctica	Acrylic resin	12:1, 40, 14	92/ 6	Du *et al.*, 2004
Aspergillus oryzae	Micro porous polymeric matrix	4:1, 40, 6	92/10	Yucel *et al.*, 2011
Thermomyces lanuginosus	Magnetic nanoparticles	6:1, 35, 18	94/5	Xie and Ma, 2009
Burkholderia fluorescens	Silica–PVA composite	3:1, 30, 36	98/6	Moreira *et al.*, 2007
Cross linking				
Pseudomonas cepacia	Cross linked with glutaraldehyde	6:1, 40, 42	92/5	Kumari *et al.*, 2007
Thermomyces lanuginosus	Polyglutaraldehyde Activated Styrene-Divinylbenzene Copolymer	6:1, 40, 40	97/7	Dizge *et al.*, 2009a
Enterobacter aerogenes	Cross linked with glutaraldehyde	4:1, 55, 48	94/7	Kumari *et al.*, 2009
Burkholderia cepacia	Cross linked with glutaraldehyde	10:1, 35, 24	98/6	Abdulla and Ravindra, 2013
Entrapment				
Staphylococcus haemolyticus	Entrapment and covalent cross-linking	6:1, 30, 10	90/10	Kim *et al.*, 2013
Pseudomonas cepacia	Entrapped sol–gel polymer	7.5:1, 35, 72	67/10	Noureddini *et al.*, 2005
Burkholderia cepacia	Phyllosilicate sol–gel	4:1, 40, 30	95/6	Hsu *et al.*, 2000
Burkholderia cepacia	Silica aerogels	6:1, 35, 24	65/8	Orcaire *et al.*, 2006

* Fatty acid alkyl esters

1.7 Conclusions

Transesterification reaction of triglycerides is often carried out by chemical catalysts for the biodiesel production. However, chemical catalysts are sensitive towards the presence of high moisture and free fatty acid contents. Lipase enzyme could be an alternative for the chemical catalysts, but it could not be adopted at industrial scale due the high cost of pure enzyme. Moreover the rate of lipase catalyzed reaction is slow in comparison to the chemical catalysts. Thus it is also worthy to find out the ways to improve the activity (reaction rate) of the lipase catalyzed transesterification reactions. Use of immobilized lipase may address the issues related with lipase stability and reusability. Different techniques were reported for the lipase immobilization on solid supports such as physical adsorption, encapsulation, entrapment, cross linking and chemical bonding with the functional group anchored on carrier. Physically adsorbed enzyme may leach out easily while catalyzing the reaction. On the other hand chemical methods of immobilization although provide better stability, but required complicated derivatization process and some time may reduce the enzyme activity. Thus selection of proper immobilization method as well as matrix is very important to prepare reusable and stable immobilized lipase to reduce the cost of enzyme catalyzed transesterification reaction.

1.8 Research gap

Literature survey suggests some of the areas for the further research:

1. Use of pure lipase for transesterification reaction is a costly affair.
2. Rate of lipase catalyzed transesterification are slow.
3. Immobilization of lipase could enhance the enzyme reusability but covalent immobilization may reduce the enzyme activity and physically adsorbed enzymes are not very stable.

1.9 Objectives

In order to enhance the enzyme activity and reusability the **following objectives** were defined for the present thesis.

i. Screening the activity of some commercially available lipases for transesterification reactions.

ii. To examine the effect of immobilized lipase on transesterification.

iii. Optimization of reaction conditions in the presence of some metal ions for effective transesterification.

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Chapter-2

Materials and methods

Abstract: This chapter includes the list of chemicals and materials, and detailed description of methods and analytical techniques which are frequently employed in present thesis.

2.1 Enzymes and Chemicals

Lipase from (E.C.3.1.1.3) *Rhizomucor miehei*, (RML, 20,000 U g^{-1}), *Candida rugosa*, (CRL, 1.2 U g^{-1}), and *Aspergillus niger*, (ANL, 187 U g^{-1}), Novozyme 435 (*Candida antarctica* lipase immobilized on acrylic resin) and Lipozyme (*Rhizomucor miehei* lipase immobilized on macroporous ion-exchange resin), pluronic copolymer 123 ($EO_{20}PO_{70}EO_{20}$), *p*-nitrophenyllaurate (*p*-NPL), 3-aminopropyl triethoxysilane (APTES), cetyltrimethyl ammonium bromide (CTAB), tetraethyl orthosilicate (TEOS), titanium isopropoxide (99%), methyl oleate (99%), methyl laurate (99%) and silica (Grade 62, surface area: 300 m^2 g^{-1}, pore volume: 1.15 cm^3g^{-1}, pore size: 150Å, particle size: 75-250 μm) were procured from Sigma-Aldrich, USA. Toluene, acetonitrile, hexane, ethyl acetate and ammonia of analytical grade were purchased from Loba Chemie, India. Oleic acid, Methanol (> 99%), ethanol, silica gel (reagent grade) for thin layer chromatography (TLC) and metal salts (AR) were procured from S.D. fine limited (India) and used as such without further purification. All vegetable oils employed for the transesterification reactions were purchased from local shops located in Patiala.

2.2 Chemical analysis of various vegetable oils

The free fatty acid (FFA) contents, saponification, and the iodine values of used Soybean oil (SO), cotton seed oil (CSO), used cotton seed oil (UCO) and jatropha oil (JO) were determined by following the methods as reported in literature (Plummer, 1988) and the moisture contents were determined by the Karl Fisher titrimetric method and respective values are listed in table 2.1.

Table 2.1. The chemical analysis of the vegetable oils.

S.No.	Feedstock	FFA value (wt%)	Moisture content (wt%)	Iodine value (mg of I_2/g)	Saponification KOH value (mg of KOH/g)
1	SO	0.5	0.27	125.4	190.0
2	CSO	0.8	0.27	121.0	192.4
3	UCO	4.0	0.26	94.7	190.4
4	JO	6.8	0.28	101.5	195.5

The fatty acid profile of the vegetable oils employed for the transesterification in present thesis, is listed in table 2.2 (Atabani *et al*., 2013).

Table 2.2. Fatty acid composition of vegetable oils employed for the transesterification in present thesis.

			CSO	**JO**	**SO**
Fatty Acid Composition* (wt% of total fatty acids)	Saturated	Myristic (C14:0)	0.4	1.4	0.5
		Palmitic (C16:0)	5	17.0	7.0-11.0
		Stearic (C18:0)	21	5.0-9.5	2.0-6.0
		Arachidic (C20:0)	6	0.3	---
	Mono unsaturated	Oleic (C18:1)	46	37-63	22.0-34.0
	Poly unsaturated	Linoleic (C18:2)	22	19-41	43.0-56.0
		Linolenic (C18:3)	---	---	5.0-11.0

*C*x*:*n*; *x* and *n* represents the number of carbon atoms and double bonds, respectively, in fatty acids.

2.3 Thin layer Chromatography (TLC)

Progress of the transesterification reaction was monitored by thin layer chromatography (TLC). The sample from the reaction mixture was withdrawn with the help

of glass capillary after appropriate time intervals. The reaction mixture thus obtained was diluted with hexane and, subjected to TLC analysis, employing silica gel as stationery and hexane/ethylacetate/acetic acid (92:7:1, v/v) as mobile phase (Katiyar and Ali, 2012). The TLC plate was developed in iodine chamber and fatty acid methyl esters (FAME) or fatty acid ethyl esters (FAEE) spot was differentiated from triglyceride spot on the basis of retention factor (R_f).

2.4 Instrumentation

2.4.1 Ultra Violet-Visible (UV-Vis) Spectrophotometer

Absorbance spectra were recorded on Perkin Elmer (Lambda–35) double-beam UV–Visible spectrophotometer.

2.4.2 Powder X-ray Diffraction (XRD)

Powder X-ray diffraction (XRD) data has been collected on Panalytical's X'Pert Pro diffractometer, operating at 40 kV and using Cu Kα radiation. The samples were scanned in the range of 2θ = 0.5-10 º at low angle with scanning speed of 2 º/min.

2.4.3 Brunauer-Emmett-Teller (BET) surface area

The surface area was determined by using the N_2 adsorption/desorption method at 77 K by the standard Brunauer-Emmett-Teller (BET) method using Micromeritics Tristar 3000 equipment. All samples were degassed at 473 K for 90 min under nitrogen atmosphere to remove the physisorbed moisture from the catalysts.

2.4.4 Field emission scanning electron microscopy (FESEM)

The surface topography of the prepared samples was analyzed with the help of field emission scanning electron microscopy (FESEM). FESEM was performed on JEOL JSM 6510LV to collect the SEM images and Field Emission Scanning Microscopy-Energy Dispersive X-ray Analysis (FESEM-EDX) studies have been performed to analyze the presence of various elements qualitatively.

2.4.5 Transmission electron microscopy (TEM)

The particle size and shape of the prepared samples have been analyzed by the HITACHI 7500 transmission electron microscopy (TEM).

2.4.6 Fourier transforms-infrared spectrophotometer (FT-IR)

The presence of various functional groups was supported by recording the Fourier Transform Infra Red (FTIR) spectra in KBr matrix on Nicolet FT-IR spectrometer (iS10, Thermo Scientific) in the range of 400-4000 cm^{-1}.

2.4.7 Fourier transform-nuclear magnetic resonance (FT-NMR)

The FAME and FAEE produced during the transesterification reactions were characterized by recording the ^{1}H and ^{13}C NMR spectra on a Bruker Avance-II (400 MHz) spectrophotometer. All spectra were recorded in $CDCl_3$ solvent and chemical shifts were expressed in parts per million (ppm) using tetramethylsilane (TMS) as an internal standard. FAME and FAEE were quantified by ^{1}H NMR technique following the equation 1 and 2, respectively (Knothe, 2001 and Ghesti *et al*., 2007).

$$\% \text{ FAMEs yield} = 100 \times (2I_a/3I_b) \quad (1)$$

where, I_a and I_b are the integration of the methoxy (3.6 ppm) and α-methylene protons (2.3 ppm), respectively in ^{1}H NMR spectrum of FAME.

$$\% \text{ FAEEs yield} = 100 \times [4(I_{c+d} - I_c)/ \{4(I_{c+d} - I_c) + 6(2I_c)\}] \quad (2)$$

where I_{c+d} = integration of glyceryl methylenic + $-OCH_2$ of ethoxy hydrogens superimposed
(4.10-4.20 ppm) and I_c = integration of glyceryl methylenic hydrogens (4.25-4.35 ppm) in ^{1}H NMR spectrum of FAEE. All reported values are an average of three experiments and an error of ±2% was observed while quantifying the FAMEs/FAEEs yield by ^{1}H NMR technique.

2.4.8 Gas chromatography-Mass spectrometry (GC-MS)

Gas chromatography-Mass spectrometry (GC-MS) of fatty acid alkyl esters were performed on Bruker GC-45X connected with Scion MS system. Gas chromatography of fatty caid methyl or ethyl esters was performed on a 15 m × 0.25 mm × 0.25 mm capillary

column (RTX -5MS sil). Samples were diluted with methanol to obtain a final concentration of 1 mg L^{-1} and one µL of this solution was injected into the GC for analysis in split/splitless mode. The flow rate of carrier gas (helium) was 1 ml/min at the initial oven temperature of 60 °C. The injection temperature was 250 °C; the oven temperature was 60 °C for 1 min hold, rose to 200 °C over 10 °C/min and then rose to 280 °C over 15 °C/min. The output from the GC column entered the ionization chamber of the mass spectrometer *via* a transfer line maintained at 260 °C. Mass Spectra (EI 70 eV, ion source temperature 280 °C, solvent delay 2.5 min) was scanned in the m/z range of 50-500 with electron impact (EI) mode of ionization. The National Institute of Standards and Technology (NIST) library match software (provided with the equipment manufacturer) was used to identify various fatty acid esters.

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Chapter-3

Effect of metal ions on the activity of native Candida rugosa lipase, Lipozyme and Novozyme 435

Abstract: In order to study the effect of metal ions, hydrolytic and transesterification activities of native *Candida rugosa* lipase (CRL) were investigated in presence of alkali (Na^{+} and K^{+}), alkaline earth (Ca^{+2} and Ba^{+2}) and transition (Cr^{+3}, Fe^{+3}, Co^{+2}, Cu^{+2} and Ni^{+2}) metal ions. Maximum enhancement in hydrolytic activity of CRL was observed by Ca^{+2}, and in transesterification activity by Cr^{+3} and Co^{+2}. Transesterification activity of commercially available immobilized lipases (Lipozyme and Novozyme 435) was also investigated, however, activity was found to decrease in presence of metal ions. The kinetics of the CRL catalyzed transesterification (methanolysis and ethanolysis) reaction was also studied, and the activation energies of methanolysis and ethanolysis were reduced from 10.13 and 10.24 kcal mol^{-1}, respectively, to 5.15 and 7.32 kcal mol^{-1}, respectively, when reactions were performed in presence of Co^{+2}.

Key words: Kinetics, Transesterification, Biodiesel, *Candida rugosa* lipase, Metal ions, Activation energy.

3.1 Introduction

Depleting resources of mineral based fuel due to heavy consumption, global warming and environmental pollution are the diverse reasons for the search of alternate and renewable energy resources. Biodiesel, chemicaly known as fatty acid alkyl esters, has emerged as an ecofriendly substitute for the convetional diesel fuel in recent past. Biodiesel has several advantages over conventional diesel fuel as it is derived from renewable resources, free from aromatic compounds, causes lower emissions of carbon monoxide, particulate matters, sulphur compounds and greenhouse gases due to its closed carbon dioxide cycle (Tan *et al*., 2010). Transesterification of naturally occuring triglycerides (vegetable oil or animal fat) with methanol in presence of chemical (e.g., NaOH, $NaOCH_3$, H_2SO_4 etc.) or bio-catalyst (lipase) yielded biodiesel and glycerol as by-product (Demirbas, 2003). At industrial scale biodiesel is mainly produced by the homogeneous base catalyzed transesterification of vegetable oils. However, homogeneous catalyst is non recyclable, non green and leads to the formation of sodium or potassium ion contaminated biodiesel (Demirbas, 2003; Kumar and Ali, 2012). Moreover homogeneous catalysts were found to be deactivated if moisture content in feedstock is > 0.3 wt% and/or free fatty acid contents (FFAs) are > 0.5 wt% and leads to the formation of soap instead of biodiesel (Demirbas and Karslioglu, 2007; Al-Zuhair

et al., 2007).

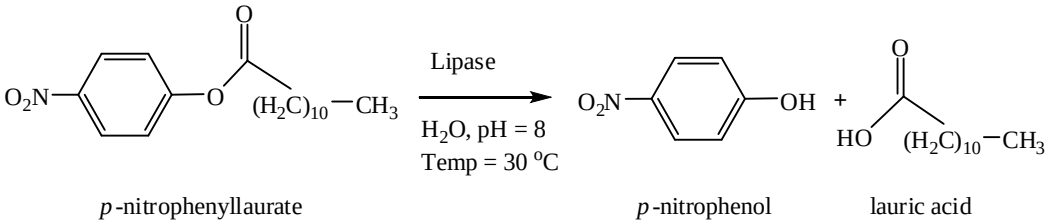
Most of the drawbacks associated with the application of chemical catalysts could be pacified by employing biocatalyst, *e.g.*, lipase (E.C. 3.1.1.3), for the transesterification of triglycerides. Direct application of lipase producing microorganisms (as whole cell), pure lipase and immobilized lipase has been well documented in literature for the transesterification of a variety of triglycerides (Ali *et al*., 2011; Katiyar and Ali, 2012). However, the biodiesel production by enzymatic method has not been adopted industrially as enzyme catalyzed reactions are relatively slow (Lu *et al*., 2008) and often not lead to the completion of the reaction (> 96.5 % FAMEs yield). Various methods have been employed in literature to improve the lipase activity, including the use of metal ions and organic molecules (Nawani *et al*., 1998; Shah *et al*., 2003). The activity of *Bacillus sp* lipase, (using *p*–nitrophenyl laurate as substrate) were found (Nawani *et al*., 1998) to increase in presence of Li^{+}, Na^{+} and Mg^{+2}, decrease in presence of Hg^{+2} and Cd^{+2} and remains uneffected by K^{+}, Ca^{+2} and Fe^{+2}. The variation in lipase activity, in presence of different metal ions, could be due to the conformational changes occurred in lipase on interacting with metal ions (Nawani *et al*., 1998).

In present chapter, effect of few metal ions (Na^{+}, K^{+}, Ca^{+2}, Ba^{+2}, Cr^{+3}, Fe^{+3}, Co^{+2}, Ni^{+2} and Cu^{+2},) on the activity of native *Candida rugosa* lipase (CRL) as well as commercially avialable immobilized lipase, *viz.*, Novozyme 435 (*Candida antarctica* lipase immobilized on acrylic resin) and Lipozyme (*Rhizomucor miehei* lipase immobilized on macroporous ion-exchange resin), has been investigated.

3.2 Experimental

3.2.1 Enzyme assay

Hydrolytic activity of lipase was estimated by performing the hydrolysis of *p*-nitrophenyllaurate (PNPL) in presence of enzyme as shown in Scheme 3.1. The hydrolysis of the PNPL yielded *p*-nitrophenol, which has a strong absorption at 405 nm (ε = 11580 L $mol^{-1}cm^{-1}$). Thus the progress of the reaction could be monitored easily by UV-Vis spectroscopy (Reetz *et al*., 2000).



Scheme 3.1. Lipase catalyzed hydrolysis of *p*-nitrophenyllaurate.

A typical assay consists of 0.4 mL CRL enzyme solution (4.0 mg mL^{-1}) in phosphate buffer (25 mM) of pH 8, 0.2 mL acetonitrilic solution of PNPL (0.5 mM) and 1.9 mL phosphate buffer (25 mM) of pH 8 in a 3.5 mL glass cuvette. The final enzyme and substrate concentration in assay was maintained 0.40 mg mL^{-1} and 0.02 mM, respectively. The course of the reaction was followed by recording the absorption spectra of assay in the wave length range of 360-450 nm over a period of 30 min as shown in Figure 3.1. Activity (1 U) of CRL is expressed as μ mol of *p*-nitrophenol produced per min by one mg of enzyme and all reported values are an average of three measurements.

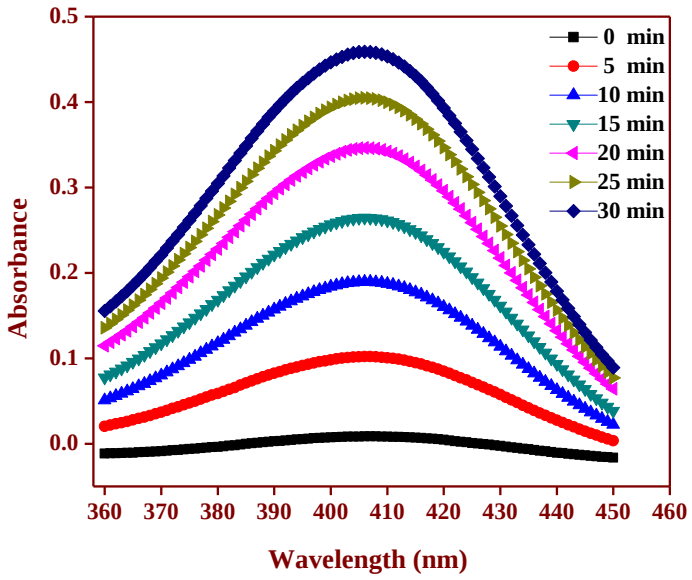


Figure 3.1. Progress of CRL catalyzed hydrolysis of *p*-nitrophenyllaurate (*p*-NPL).

To evaluate the effect of metal ions (Na^{+}, K^{+}, Ca^{+2}, Ba^{+2}, Cr^{+3}, Fe^{+3}, Co^{+2}, Ni^{+2} and Cu^{+2}) on lipase activity, 0.4 ml aqueous solution (10 mM) of desired metal was added to the above mentioned assay, maintaining the final volume of 2.5 ml.

Blank experiments have also been performed in presence of metal ions but without adding CRL to the assay. Under such conditions no hydrolysis of PNPL was observed, thus metal ion itself remain silent towards the hydrolysis reaction.

Transesterification activity of CRL was evaluated by employing cotton seed oil (CSO) as substrate in presence of methanol or ethanol. Prior to the reaction, in order to activate the pure enzyme, 50 mg of native CRL was soaked with 1 mL deionized water for 30 min (Al-Zuhair, 2008). In a typical reaction, 25 mL round bottom flask was charged with 50 mg of activated CRL, 1 mL metal solution (10 mM), 1.2 ml of CSO and 0.55 ml methanol or 0.81 ml ethanol (alcohol to oil molar ratio = 12:1) and reaction mixture was stirred for desired duration at 40 ºC. The reactions were continued till the complete transesterification of oil into corresponding monoalkyl esters (99 %) was achieved. The course of the reaction was followed by withdrawing the samples from the reaction mixture after every 4 h and quantifying the fatty acid methyl esters (FAMEs) or fatty acid ethyl esters (FAEEs) by ^{1}H NMR technique (Knothe, 2001; Ghesti *et al*., 2007) following the equation 1 and 2, respectively.

$$\% \text{ FAMEs yield} = 100 \times (2I_a/3I_b) \quad (1)$$

where, I_a and I_b are the integration of the methoxy (3.7 ppm) and α-methylene protons (2.3 ppm), respectively in ^{1}H NMR spectrum of FAMEs.

$$\% \text{ FAEEs yield} = 100 \times [4(I_{c+d} - I_c)/\{4(I_{c+d} - I_c) + 6(2I_c)\}] \quad (2)$$

where I_{c+d} = integration of glyceryl methylenic + $-OCH_2$ of ethoxy hydrogens superimposed (4.10-4.20 ppm) and I_c = integration of glyceryl methylenic hydrogens (4.25-4.35 ppm), in ^{1}H NMR spectrum of FAEEs.

To evaluate the effect of metal ions (Na^+, K^+, Ca^{+2}, Ba^{+2}, Cr^{+3}, Fe^{+3}, Co^{+2}, Ni^{+2} and Cu^{+2}) on transesterification activity of CRL, Novozyme 435 and Lipozyme RM-IM, 1 ml aqueous solution (10 mM) of desired metal was added to the enzyme assay.

Blank experiments were carried out following the same assay as mentioned above but using only metal ion solution in place of CRL. Under such condition negligible conversion (< 5 %) of oil to fatty acid methyl esters were observed, to maintain that metal ions remain almost silent towards the transesterification reaction.

Cotton seed oil derived FAMEs **^{1}H-NMR** ($CDCl_3$, δ ppm): 5.3 (m, -C**H**=C**H**-), 3.6 (s, -OC**H_3**), 2.7 (m, -CH=CH-C**H_2**-CH=CH-), 2.3 (m, -C**H_2**-CO-), 2.0 (m, -C**H_2**-CH=CH-), 1.6-1.25 (m, -(C**H_2**)$_n$-), 0.95 (m, -CH=CH-C**H_3**), 0.87 (m, -CH_2-C**H_3**). **^{13}C-NMR** ($CDCl_3$, δ ppm): 174.1 (-**C**O-CH_2-), 129.9 (-**C**H=**C**H-), 77.1 ($CDCl_3$), 51.2 (-O**C**H_3), 34.1 (-CO-**C**H_2-), 31.9 (ω3 -**C**H_2-), 29.66-29.08 (-CH=CH-**C**H_2-, -**C**H_2-), 27.2 (-CH=CH-**C**H_2-CH=CH-), 25.6-24.80 (-CO-CH_2-**C**H_2-), 22.70, 22.47 (ω2 -**C**H_2-) and 14.16 (ω1 -**C**H_3).

Cotton seed oil derived FAEEs **^{1}H-NMR** ($CDCl_3$, δ ppm): 5.3 (m, -C**H**=C**H**-), 4.2 (q, -OC**H_2**-C**H_3**), 2.7 (m, -CH=CH-C**H_2**-CH=CH-), 2.3 (m, -C**H_2**-CO-), 2.0 (m, -C**H_2**-CH=CH-), 1.6-1.25 (m, -(C**H_2**)$_n$-), 0.95 (m, -CH=CH-C**H_3**), 0.87 (m, -CH_2-C**H_3**). **^{13}C-NMR** ($CDCl_3$, δ ppm): 174.09(-**C**O-CH_2-), 129.9 (-**C**H=**C**H-), 77.1 ($CDCl_3$), 61.5 (-O**C**H_2 -CH_3), 34.1 (-CO-**C**H_2-), 31.9 (ω3 -**C**H_2-), 29.66-29.08 (-CH=CH-**C**H_2-, -**C**H_2-), 27.2 (-CH=CH-**C**H_2-CH=CH-), 25.6-24.80 (-CO-CH_2-**C**H_2-), 22.70, 22.47 (ω2 -**C**H_2-) and 14.16 (ω1 -**C**H_3).

3.3 Results and discussion

3.3.1 FAME and FAEE characterization

The proton NMR technique is employed to characterize and quantify the fatty acid methyl or ethyl esters. The ^{1}H NMR spectrum of cottonseed oil shows a multiplet at 4.15–4.34 and 5.25 ppm due to the presence of glyceridic protons (Figure 3.2a). The peaks appearing at 0.8–2.8 ppm are due to the presence of saturated hydrocarbon protons of fatty acids in FAME/FAEE as well as CSO. The unsaturated protons (-CH=CH-) of fatty acid carbon chain in CSO as well as FAMEs/FAEEs appear at 5.35 ppm. Upon methanolysis a new peak appears at 3.6 ppm due to –OCH_3 protons (Figure 3.2b), and peaks corresponding to the glyceridic protons were no longer found, to support the formation of methyl esters of oil.

Similarly, in proton NMR spectrum of FAEE (Figure 3.2c) appearance of a quartet at 4.09–4.16 ppm, due to the methylenic protons (–OCH_2–) of ester group, indicate the formation of ethyl esters from CSO.

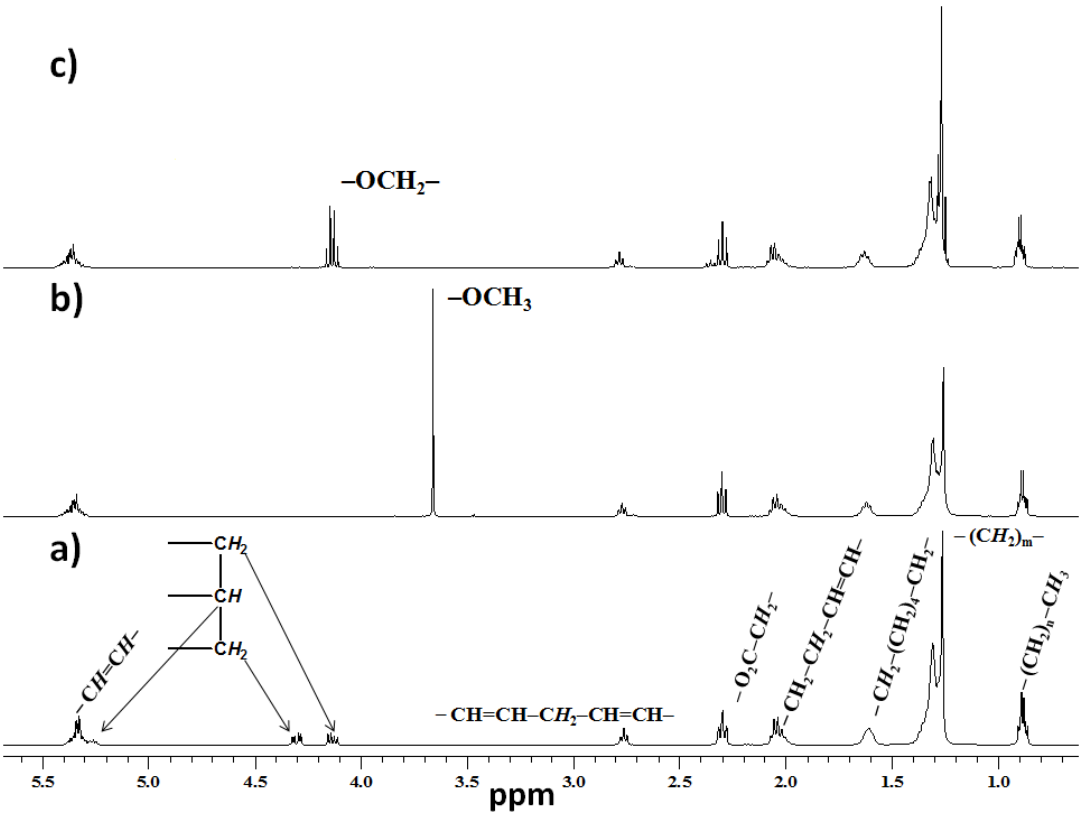


Figure 3.2. Comparison of ^{1}H-NMR spectra of (a) cottonseed oil (b) FAME and (c) FAEE

In ^{13}C-NMR spectrum of CSO, peaks due to glyceridic carbon appear at 61.2 and 69.1 ppm, as shown in Figure 3.3a. The formation of FAME, and FAEE was supported by the appearance of a peak at 51.2 and 61.1 ppm, respectively due to $–OCH_3$ and $–O\boldsymbol{CH_2}CH_3$ groups, respectively. The signals due to carbonyl, unsaturated and saturated carbons appeared at 172-174, 127-130, and 13-34 ppm, respectively in CSO as well as FAEE and FAME. The peaks corresponding to the glyceridic carbons were no longer found in the ^{13}C-NMR spectrum of FAME as well as FAEE, to support the conversion of CSO into respective alkyl esters.

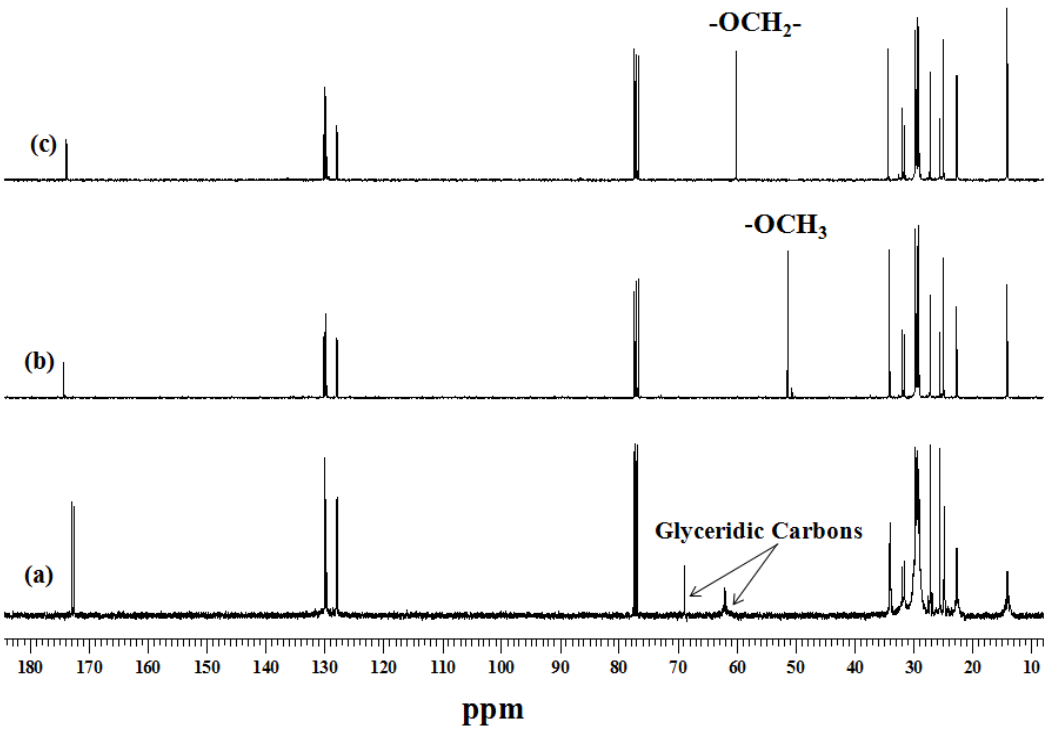


Figure 3.3. Comparison of ^{13}C-NMR spectra of (a) cottonseed oil (b) FAME and (c) FAEE.

The GC-MS analysis was used to study the chemical composition of cotton seed oil derived fatty acid methyl or ethyl esters. Five major peaks, corresponding to a fatty acid methyl or ethyl esters, were observed in the gas chromatogram (Figure 3.4a and 3.5a). The Mass spectra corresponding to each peak was recorded (Figure 3.4b-f and 3.5b-f) and respective ester was identified from the NIST library match software. The major peak in all mass spectra of saturated FAMEs was observed at m/z 74 due to the well-known McLafferty rearrangement process (Tariq *et al*., 2011). Another characteristic peak in case of FAMEs was observed at m/z [M-31] due to the loss of methoxy group. In case of unsaturated fatty acid methyl or ethyl esters, characteristic fragmentation patterns possessing peaks at m/z 55 (base peak), [M-31] due to loss of methoxy group and [M-74] due to loss of McLafferty ion (Tariq *et al*., 2011) were observed.

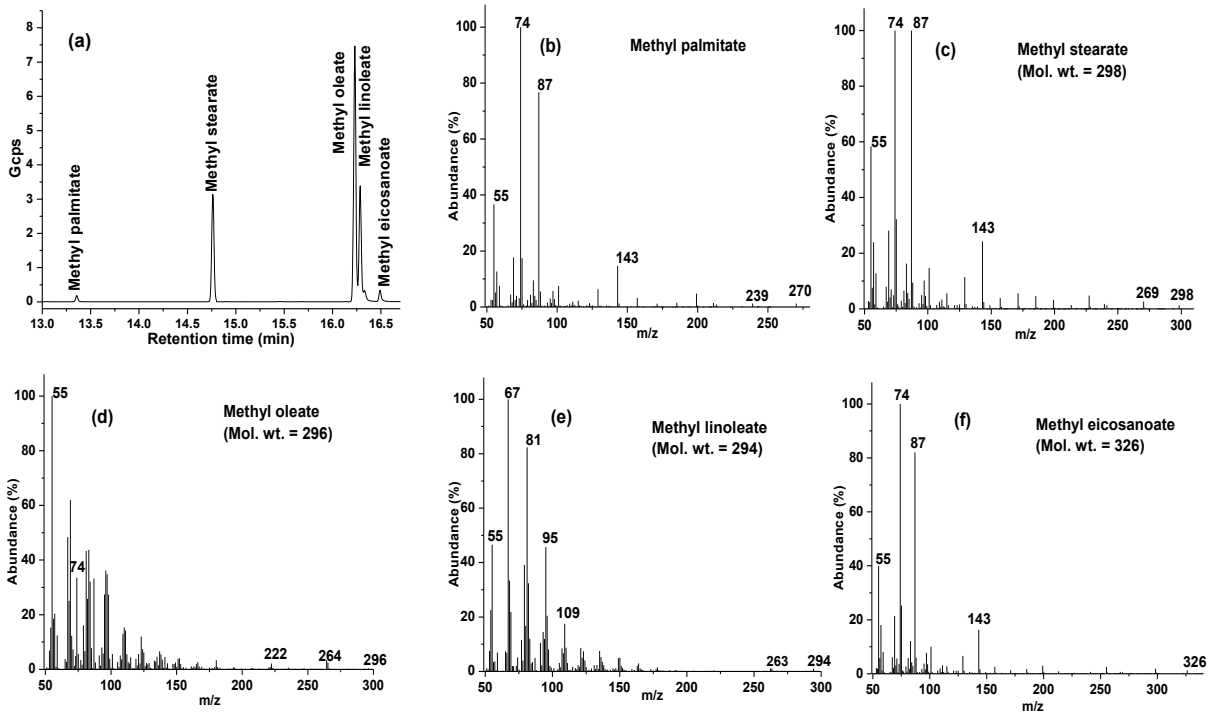


Figure 3.4. (a) GC- (b-f) MS of cotton seed oil derived FAMEs

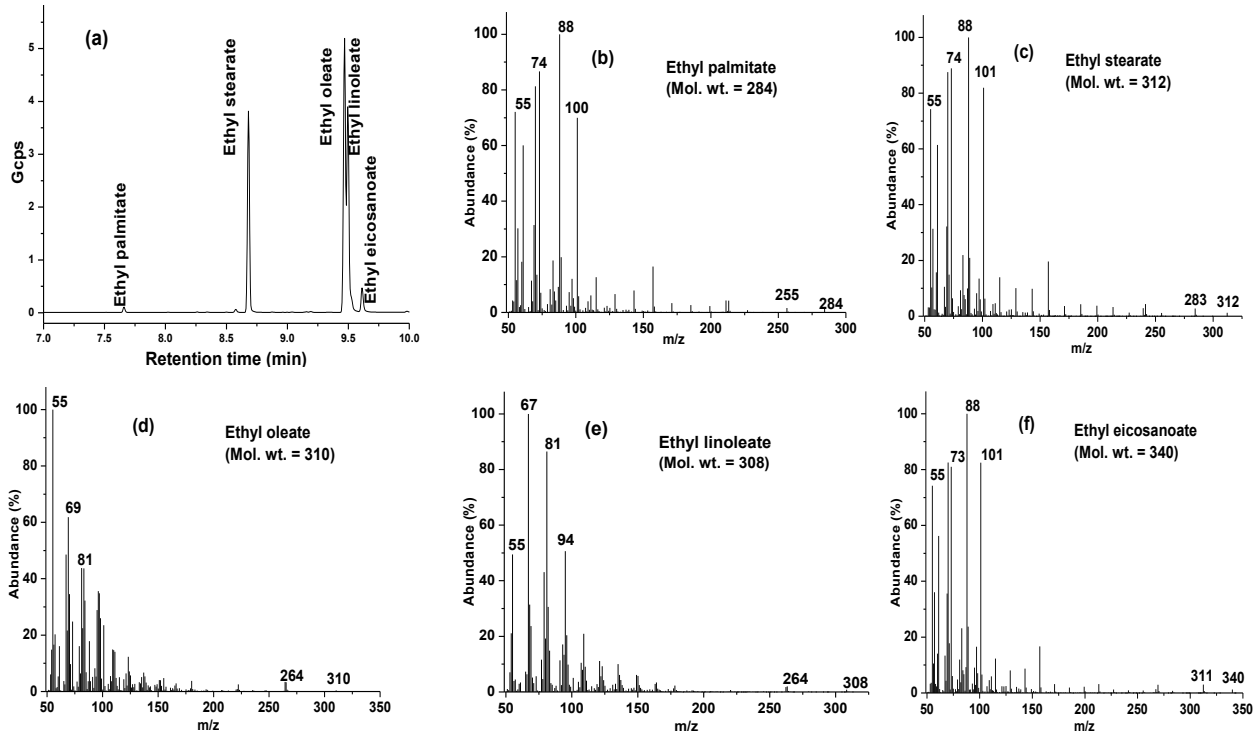


Figure 3.5. (a) GC- (b-f) MS of cotton seed oil derived FAEEs

3.3.2 Effect of metal ions on the activity of native CRL

3.3.2.1 Hydrolytic activity

Lipase is an ubiquitous enzyme which could catalyze the hydrolysis, esterification and transesterification reactions in biological systems. During hydrolysis and transesterification, lipase acts on ester bond (OC—O) and it is expected that metal ions should influence both type of activities. As would be discussed in subsequent sections that studied metal ions were found to effect lipase catalyzed hydrolysis as well as transesterification, but not to the similar extent.

The activity of the pure lipase was observed as 10.61 U (100% activity) and same activity was used as a base while calculating the relative activity of CRL in presence of metal ions as given in Table 3.1. The metal ions employed in present study were selected from three different groups: – (i) alkali metals (Na^{+} and K^{+}), (ii) alkaline earth metals (Ca^{+2} and Ba^{+2}), and (iii) transition metals (Cr^{+3}, Fe^{+3}, Co^{+2}, Ni^{+2} and Cu^{+2}). In order to nullify the effect of counter anion, all metal ions were taken as their nitrate salts.

The metal ions on the basis of their effect on CRL activity (Table 3.1) could be divided in three categories *viz.*, (a) Ca^{+2}, Fe^{+3}, Cu^{+2} and Co^{+2} were found to enhance the activity, (b) K^{+} and Ba^{+2} remain silent towards the activity and (c) Na^{+}, Ni^{+2} and Cr^{+3} were found to reduce the CRL activity.

Table 3.1. Comparison of the hydrolytic activity of CRL in presence of various metal ions.

Metal ions	Nil	Na^{+}	K^{+}	Ca^{+2}	Ba^{+2}	Cr^{+3}	Fe^{+3}	Co^{+2}	Ni^{+2}	Cu^{+2}
Relative activity (%)	100	82	96	153	109	44	127	136	72	145

In literature no consistent trend has been reported (Sharon *et al*., 1998; Shangguan *et al*., 2011) regarding the effect of metal ions on lipase hydrolytic activity. The effect varies from metal to metal and even same metal ion was found to effect the lipase activity to different extent if lipase source was changed (Unsal *et al*., 2011). However, in most of the reports, as also observed in present study, Ca^{+2} were found to enhance the lipase hydrolytic activity (Ma *et al*., 2006; Sharon *et al*., 1998).

3.3.2.2 Transesterification (methanolysis and ethanolysis) activity

Fatty acid methyl or ethyl esters are usually prepared from the transesterification reaction of triglycerides (oil or fat) with methanol or ethanol in presence of a chemical catalyst. Lipase catalyzed transesterification was found to be relatively slow and often such reactions either required longer reaction duration (upto 105 h) or leads to the incomplete conversion of oil into corresponding monoalkyl esters (Yagiz *et al*., 2007). Hence, it is worth to find out the methods which could enhance the lipase activity and yield the complete conversion of oil into corresponding monoalkly esters in lesser reaction duration.

The activity of pure lipase was compared with that of metal activated one on the basis of their respective turn over frequencies (TOF) for the transesterification of CSO with methanol or ethanol. As could be seen from Figure 3.6, the methanolysis activity of CRL was enhanced by all the metal ions investigated as supported by the increase in TOFs. However, in case of ethanolysis, all metal ions employed were not found to enhance the TOF and in presence of Na^{+}, Ba^{+2} and Ni^{+2} the TOFs were even found to be reduced. On the other hand, maximum ethanolysis activity (6.75 h^{-1}) was observed in presence of Cr^{+3} and Co^{+2}. Hence, addition of Cr^{+3} and Co^{+2} could enhance the activity of CRL more than twice, towards the transesterification of CSO with methanol as well as ethanol.

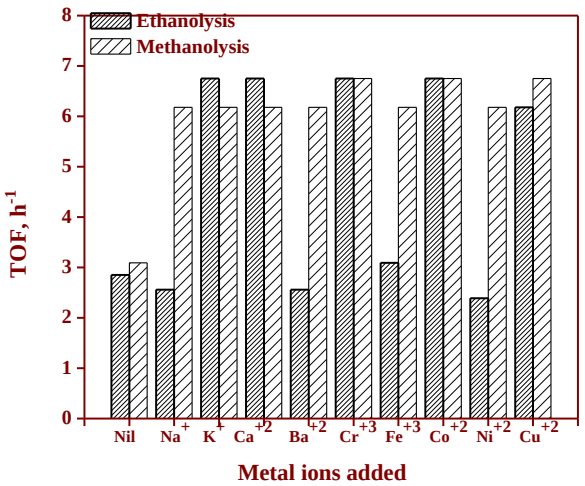


Figure 3.6. Comparison of the transesterification activity of pure lipase with that of metal activated one. Reaction conditions: methanol or ethanol/oil molar ratio = 12:1, enzyme = 5 wt% of oil, metal = 1 mL (10 mmol) and, stirring speed = 250 rpm; TOF = rate of conversion of CSO into FAMEs or FAEEs (moles/h)/moles of lipase.

On the basis of comparison between hydrolytic and transesterification activity in presence of metal ions it was observed that Na^{+}, K^{+}, Cr^{+3} and Ni^{+2}, were found to influence both the activities to the different extent. Hence, on the basis of hydrolytic activity alone it would be not be possible to predict the effect of a metal ion on transesterification activity. Moreover, even in case of transesterification, except Cr^{+3} and Co^{+2} all other metal ions were found to effect CRL catalyzed ethanolysis and methanolysis to a different extent. Hence, influence of metal ions on various lipase activities could not be generalized as it was also found a function of substrate employed for the reaction.

3.3.3 Effect of metal ions on the transesterification activity of immobilized enzymes

Transesterification reaction is important from commercial point of view as it is used for the production of biodiesel, at industrial scale. Mainly this reaction is catalyzed by chemical catalysts as lipase catalyzed transesterification is not viable at industrial scale due to extremely high cost of enzyme. Recycling of the immobilized lipase could reduce the effective cost of lipase and hence, in recent past application of immobilized enzyme for the transesterification has gained significant attention (Kim *et al*., 2013). Novozyme 435 and Lipozyme are the commercially available immobilized lipase, frequently employed in literature (Xu *et al*., 2003) for the transesterification reactions. To study the effect of metal ions on the transesterification activity, both enzymes were separately employed in reaction assay in place of CRL. As could be seen from Figure 3.7a and 3.7b, the metal ions were not found to influence the activity of these enzymes positively and in fact in all the cases activity was reduced by the presence of metal ions.

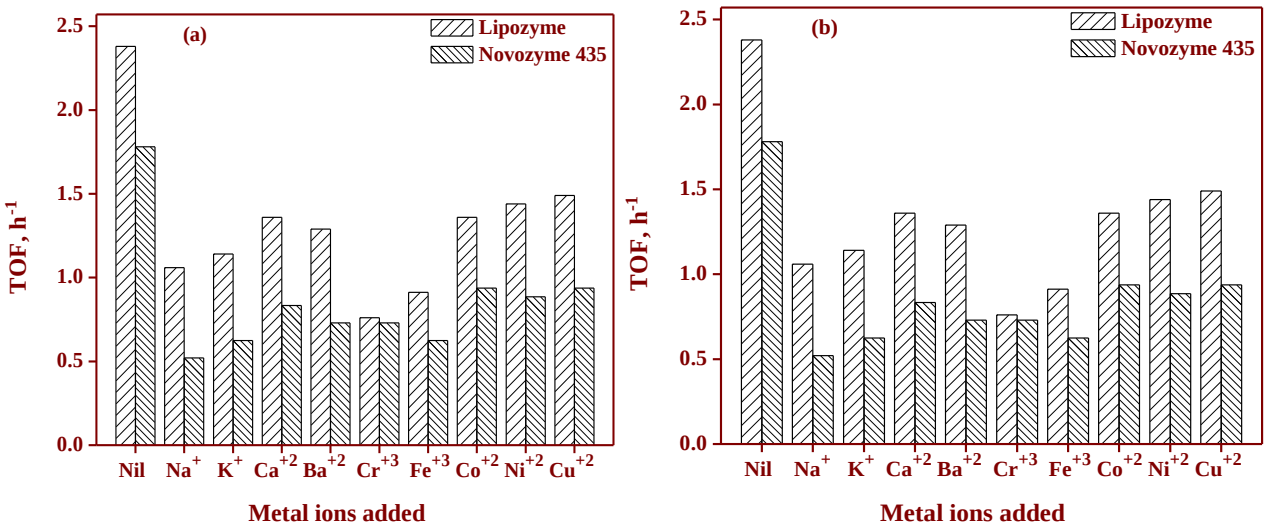


Figure 3.7. Comparison of the transesterification activity of Lipozyme and Novozyme 435 with that of metal activated one (a) Methanolysis (b) Ethanolysis. Reaction conditions: alcohol/oil molar ratio = 9:1, enzyme = 5 wt% of oil, metal = 1 mL (10 mmol) and, stirring speed = 250 rpm; TOF = rate of conversion of CSO into FAMEs or FAEEs (moles/h)/moles of lipase.

3.3.4 Kinetic study

As evident from the comparison of TOFs, Cr^{+3} and Co^{+2} were found to enhance the CRL catalyzed ethanolysis as well as methanolysis activity to the maximum and similar extent among the metal ions investigated. The increase in activity could be due to the reduction in activation energy when reaction was performed in presence of these metal ions. In order to evaluate the activation energy, the kinetics of the lipase catalyzed transesterification in presence of Co^{+2} was studied as an example.

During transesterification of triglycerides, in every step an alcohol molecule is consumed and one fatty acid alkyl ester molecule is produced along with diglyceride, moglyceride and glycerol molecules in first, second and third step, respectively. Thus, theoretically one triglycerides molecule required three molecules of alcohol for the complete transesterification, and hence the reaction should follow fourth order kinetic equation. Nevertheless, usually transesterification reactions are performed in presence of excess alcohol. Hence, rate of reaction was found to be independent from the alcohol concentration and follow the pseudo-first order kinetic equation (Song *et al.*, 2011). In present study CRL catalyzed transesterification of CSO were performed in presence and absence of Co^{+2}, employing 12:1 methanol or ethanol to oil molar ratio at 20, 25, 30, 35, 40

and 50 ºC reaction temperatures. In order to study the reaction kinetics pseudo-first order kinetic equation was employed as given in equation 3.

$$kt = \ln \frac{1}{1-X_e} \quad (3)$$

where, X_e is the fraction of FAEEs or FAMEs produced, t is the time in h and k is the pseudo–first order rate constant. The values of (pseudo) first order rate constants in presence of Co^{+2} were calculated from $-\ln(1-X_{me})$ or $-\ln(1-X_{ee})$ *vs* time plot as shown in Figure 3.8.

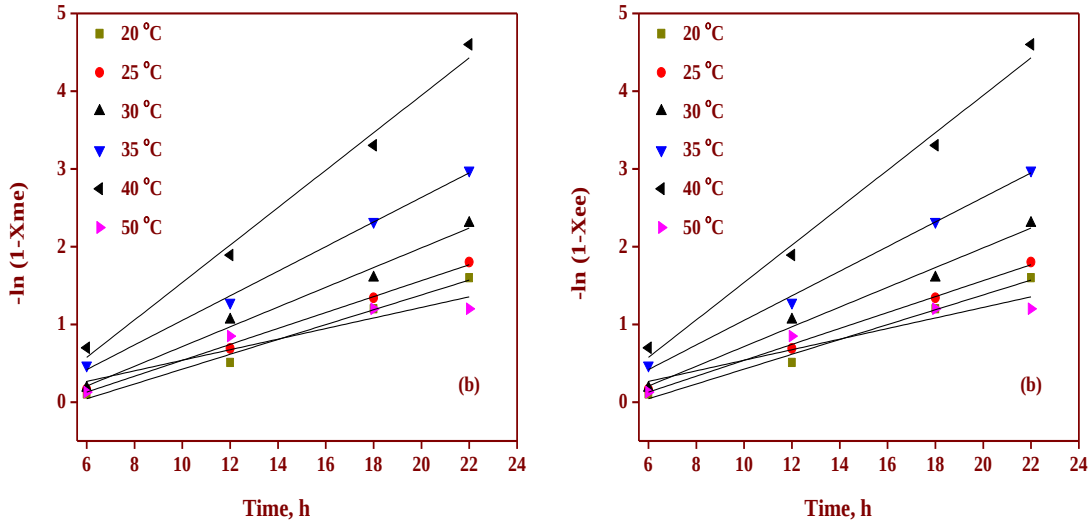


Figure 3.8. Plot of (a) –ln (1- X_{me}) and (b) –ln (1- X_{ee}) against time (t) at different reaction temperatures; where X_{me} and X_{ee}, are the fractions of FAMEs and FAEEs, respectively.

The values of rate constants were found to increase regularly as the reaction temperature was increased from 20 to 40 ºC. However, a further increase in reaction temperature (50 ºC) reduces the enzyme activity, may be due to the partial enzyme denaturation at this temperature.

The Arrhenius equation 4 has been employed to calculate the activation energy (E_a) for the transesterification reactions performed in presence and absence of Co^{+2}.

$$k = A.e^{-E_a/RT} \quad (4)$$

where, R is the gas constant, A is the pre-exponential factor and T is the reaction temperature in K. A plot between lnk and 1/T, yielded a straight line as given in Figure 3.9.

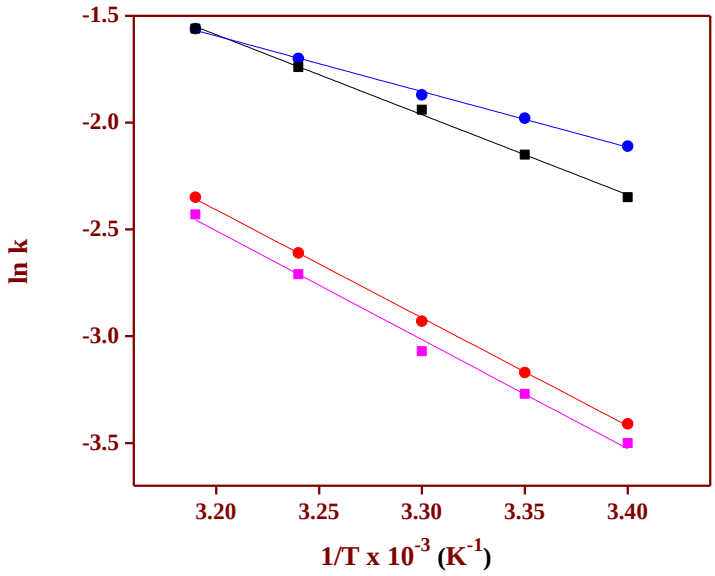


Figure 3.9. Arrhenius plot for the lipase catalyzed transesterification of cotton seed oil. ●, ○ = methanolysis in presence and absence of Co^{+2}, respectively; and ■, □ = ethanolysis in presence and absence of Co^{+2}, respectively.

The values of E_a of CRL catalyzed ethanolysis and methanolysis reactions in presence and absence of Co^{+2} were calculated from the Arrhenius plot and summarized in Table 3.2. The comparison of E_a supports the significant reduction in activation energies when reactions were carried out in presence of Co^{+2}. The increase in reaction rate, in presence of Co^{2+}, is further supported by the reduction in time, required for the completion of reaction (Table 3.2).

Table 3.2. Comparison of the activation energies (E_a) and time (t) required for the complete transesterification for the lipase catalyzed transesterification in presence and absence of Co^{+2}

	No metal ion		**In presence of Co^{+2}**	
	Methanolysis	**Ethanolysis**	**Methanolysis**	**Ethanolysis**
(E_a, kcal mol^{-1})	10.13	10.24	5.15	7.32
(t, h)*	48	52	22	22

* time required for the complete methanolysis or ethanolysis of CSO.

Accordingly it is safe to assume now that metal ions which were found to affect the lipase activity positively could have reduced the activation energy of the reactions. However, the exact nature of metal–lipase interaction and effect of these interactions on lipase active site is a matter of further investigation.

3.4 Conclusions

Hydrolytic and transesterification activity of *Candia rugosa* lipase has been examined in presence of few metal ions. Out of these metal ions Ca^{+2}, Ba^{+2}, Fe^{+3}, Co^{+2} and Cu^{+2} were found to enhance the activity of lipase. All the metal ions, studied, were found to enhance the methanolysis activity, and Cr^{+3} and Co^{+2} were found to enhance the methanolysis as well as ethanolysis to the maximum and similar extent. Time required for the complete methanolysis and ethanolysis of cotton seed oil with CRL was reduced from 48 and 52 h, respectively to 22 h, when lipase was employed in presence of Co^{+2}. On the other hand, a negative effect on transesterification activity of immobilized lipases (Lipozyme and Novozyme) was observed due to the presence of metal ions. Thus, in CRL catalyzed transesterification Cr^{+3} or Co^{+2} could be added to the assay in order to produce the biodiesel in relatively shorter reaction duration.

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Chapter-4

Immobilization of Candida rugosa lipase on MCM-41, SBA-15 and Ti/SiO_2 for the transesterification of cotton seed oil

Abstract: Present study demonstrated the preparation of MCM-41, SBA-15 and Ti/SiO_2 as a support for the immobilization of *Candida rugosa* lipase (CRL) by the physical adsorption technique. The lipase immobilized MCM-41, SBA-15 and Ti/SiO_2 were characterized by powder X-ray diffraction, scanning and transmission electron microscopic and Fourier transform infrared techniques. Lipase immobilization on mesoporous support was found to be a function of pH of medium and maximum immobilization on MCM and Ti/SiO_2 was observed at pH 6, while on SBA-15 at pH 3. Finally the immobilized enzymes were employed for the transesterification of cotton seed oil and reaction was found to be a function of pH, reaction temperature and methanol/oil molar ratio. The reusability study of the immobilized enzymes was also performed and only partial conversion was achieved in second and third catalytic run.

Key words: Transesterification, Biodiesel, immobilization, *Candida rugosa* lipase, MCM-41, SBA-15 and Ti/SiO_2.

4.1 Introduction

As discussed in previous chapter the application of biocatalyst, such as lipase, for biodiesel production could pacify problems associated with chemical catalysts (Lara Pizarro and Park, 2003; Severac *et al*., 2011). Several reports are available in literature regarding the application of lipase as biocatalyst for biodiesel production (Al-Zuhair, 2008; Tan *et al*., 2010). However, use of pure lipase for the production of biodiesel has not been adopted at industrial scale due to its higher cost (Cao, 2005; Salis *et al*., 2008). Although rate of lipase catalyzed reaction could be enhanced by performing the reactions in presence of metal ions, however, the enzyme could not be isolated and recycled after the completion of the reaction. To reduce the effective cost of enzyme catalyzed transesterification, lipase producing microorganism or native lipase has been immobilized on carriers (Fukuda *et al*., 2001, Ali *et al*., 2011). In literature a variety of carriers, *e.g.,* acrylic resin, textile membrane, polypropylene, celite, mesoporous silica, and diatomaceous earth, have been employed for the lipase immobilization by physical adsorption technique (Tan *et al*., 2010). Silica-based porous material, because of their high surface area and tunable pore diameter, has also gained popularity, in recent past (Ying *et al*., 1999), as support material for the immobilization of enzymes.

In present study three different supports such as MCM-41, SBA-15 and Ti/SiO_2 have been employed to immobilize the CRL by physical adsorption method. The prepared solid biocatalyst has been employed for the transesterification of cotton seed oil with methanol to produce FAMEs. The reusability of the immobilized enzymes was also evaluated.

4.2 Experimental

4.2.1 Enzyme solutions

The solution of CRL of 3.4 mg/ml concentration was prepared by shaking 68 mg of enzyme in 20 mL buffer solution (25 mM) of desired pH (3-10) at 25 ºC. Acetate, phosphate and tris/HCl were used for making the buffer solutions of pH 3-5, 6-8, and 9-10, respectively. The CRL solutions thus obtained were centrifuged and clear supernatant was collected and stored at 4 ºC for further use.

4.2.2 Synthesis of support

Mesoporous MCM-41 material was synthesized by following the reported procedure (Kumar *et al*., 2001) with slight modification. In a typical preparation method, 2.4 g CTAB was dissolved in 120 mL of deionized water in a 250 ml round bottom flask equipped with a magnetic stirrer. The reaction mixture was stirred at room temperature to obtain a clear homogenous solution and to this 8 ml ammonia (33%; v/v) and 10 ml TEOS was added to yield a gel. The final molar composition of the gel was 1 M TEOS: 1.64 M NH_3: 0.15 M CTAB: 126 M H_2O.The reaction mixture was aged at 25 ºC for 12 h, filtered and washed consecutively with deionized water and ethanol. The semi-solid product thus obtained was dried at 120 ºC for 12 h and finally calcined at 550 ºC for 5 h to obtain mesoporous MCM-41 material.

Mesoporous SBA-15 was prepared by the literature reported sol-gel method (Katiyar and Ali, 2006, Cao *et al*. 2009) and using non-ionic triblock copolymer, pluronic acid, as template and CTAB as co-surfactant and cyclohexane as swelling agent. In a typical preparation, 3 g of pluronic copolymer (P123) was dissolved in 60 ml, 1.5 M HCl. In another round bottom flask, CTAB (0.6 g) and cyclohexane (1.9 mL) was mixed with deionized water (25 mL). Both the mixtures were mixed together in a 250 mL round bottom flask equipped with a hot plate, magnetic stirrer and water cooled condenser, and to this 20

mL ethanol was added. Then, TEOS (10 mL), was added drop by drop to this solution and resulted mixture was stirred at 35 ºC for 45 min. This mixture was transferred in a PTFE lined autoclave and heated at 80 ºC under static condition for 10 h. After the stipulated time the autoclave was cooled to the room temperature, and mixture was transferred to the beaker and dried at 100 ºC. The white solid thus obtained was isolated, washed with deionized water repeatedly, dried at room temperature and finally calcined at 600 ºC for 5 h to obtain SBA-15.

Titanium-loaded silica (Ti/SiO_2) was prepared by the chemical method by following the literature-reported procedure (Sanchez *et al*., 2000; Kumar and Ali, 2012) with slight modification. A two-neck round-bottom flask equipped with a water-cooled condenser, oil bath, and magnetic stirrer was charged with toluene (75 mL) and titanium isopropoxide (0.59 g) and heated at the boiling point of toluene (110 ºC). To this suspension, 5 g of silica was added, and resulting mixture was stirred at the same temperature for 5 h. The solid formed was isolated by filtration, washed twice with hot toluene, subsequently dried at 100 ºC, and finally calcined at 650 ºC for 8 h to obtain Ti/SiO_2.

4.2.3 Enzyme immobilization by physical adsorption

Immobilization of lipase on MCM-41 was carried out by physical adsorption technique in 50 mL conical flask. In a typical experiment, 20 mL enzyme solution (3.4 mg/ml) was agitated with 80 mg MCM-41 at a speed of 160 shakes/min at 20 ºC for 4 h. The amount of immobilized lipase was determined by recording the absorption spectra of supernatant after every 30 min at 257 nm and following the literature reported (Bai *et al*., 2010) method. The amount of CRL immobilized over MCM-41 was calculated by substituting the appropriate values in equation 1:

$$P = \{(C_0-C_1)\, V\}/\, W \qquad (1)$$

where, P is the amount of CRL adsorbed on support (mg/g), C_0 and C_1 are the initial and final concentration (after adsorption) of CRL solutions, V is the total volume (mL) of the reaction medium and W is the weight (g) of support.

The resulting reaction mixture was then centrifuged at 8000 rpm for 20 min to separate CRL-MCM-41 from the supernatant. Similar procedure was employed to immobilize the CRL on SBA-15 and Ti/SiO_2 to obtain CRL-SBA-15 and CRL-Ti/SiO_2,

respectively. The immobilized enzymes were finally dried at 30 ºC, stored at 4 ºC and used for the transesterification of cotton seed oil with methanol.

4.2.4 Activity of free and immobilized lipase

In order to compare the lipase activity, the hydrolysis of *p*-nitrophenyllaurate (*p*-NPL) was performed and reaction was followed by measuring the absorbance at 405 nm. The complete detail of the method is described in chapter 3.

The enzyme assay for the native CRL consists of 0.3 mL enzyme solution (3.4 mg/mL) in phosphate buffer (25 mM), 0.2 mL ethanolic solution of *p*-NPL (0.3 mM) and 2.5 mL phosphate buffer (25 mM) of pH 8 in a glass cuvette. The final enzyme and substrate concentration in assay was maintained 0.34 mg/mL and 0.02 mM, respectively. Lipase activity (1 U) was defined as μmol of *p*-nitrophenol produced per min by per mg of enzyme and activity of free lipase was found to be 8.62 U.

For immobilized enzyme, 68 mg of CRL-MCM-41 in 46.67 mL phosphate buffer (25 mM) and 3.33 mL ethanolic solution of *p*-NPL (0.3 mM) were mixed in a conical flask. The mixture was shaken in orbital shaker (200 shakes/min) at room temperature and to monitor the progress of the reaction one mL sample were withdrawn after every 10 min, filtered and analyzed by UV-Vis spectroscopy by measuring the absorbance at 405 nm. The activity (1 U) of immobilized lipase is given as μmol of *p*-nitrophenol produced per min by per mg of immobilized enzyme and found to be 6.74 U for CRL-MCM-41.

4.2.5 Transesterification of cotton seed oil

Transesterification reactions of cotton seed oil with methanol was performed in a two neck 25 ml round bottom flask equipped with a water cooled condenser, water bath and a magnetic stirrer. In a typical transesterification reaction round bottom flask was charged with 1.0 g cotton seed oil, 50 mg of CRL-MCM-41 and 2 mL phosphate buffer (25 mM) of pH 8. To this, 0.55 mL methanol was added in three equally divided amounts (final methanol to oil molar ratio = 12:1) after every 4 h of reaction duration and reaction mixture was stirred for 48 h at 40 ºC. Blank experiments were also carried out following the same assay as mentioned above but using MCM-41 in place of CRL-MCM-41. Under such condition negligible conversion (< 5%) of oil to FAMEs were observed.

The progress of the reaction was monitored by thin layer chromatography (TLC) by withdrawing the sample from the reaction mixture with the help of glass capillary after every 4 h. The reaction mixture thus obtained was diluted with hexane, subjected to TLC analysis by using silica gel as stationery and hexane/ethylacetate/acetic acid (92:7:1, v/v) as mobile phase. The TLC plate was developed in iodine chamber to visualize FAMEs (R_f = 0.8) and oil (R_f = 0.5) spots. After the completion of the reaction, the reaction mixture was filtered and liquid phase was kept in a separating funnel. The upper FAMEs layer was isolated and characterized and quantified by ^{1}H-NMR technique by following the literature reported procedure (Knothe, 2001) as discussed in chapter 2 and 3.

4.3 Results and discussion

4.3.1 Characterization of immobilized lipase

4.3.1.1 X-ray diffraction study

The low angle XRD patterns of the MCM-41, SBA-15 and Ti/SiO_2 and respective lipase supported materials were compared in Figure 4.1. In the XRD patterns of the MCM-41, peaks at 2θ ~ 0.3, 1.9, and 2.5º, corresponds to the d-values of 34.8, 19.9 and 18.3 Å, respectively. Same peaks could be indexed as (100), (110) and (200) reflections planes, respectively. This type of diffraction patterns is consistent with the hexagonal structure of prepared MCM-41 material with *p6mm* symmetry (Hua *et al.*, 2005). Upon lipase immobilization, the amorphous nature of MCM increases as only one peak at ~ 0.6º was observed in the diffraction patterns of CRL-MCM-41 material.

The XRD pattern of SBA-15 showed four well resolved peaks at 2θ ~0.78º, 0.97º, 1.32º and 1.64º and same could be indexed as (100), (110), (200) and (210) reflections planes, respectively. This type of diffraction pattern support the long range ordered structure in the prepared SBA-15 material and also in agreement with the 2-D hexagonal structure with *p6mm* symmetry (Katiyar *et al*., 2006). Due to the immobilization of CRL, the intensity and number of XRD peaks of SBA-15 was found to reduce and only two broad peaks at *2θ* ~ 1.22º and 2.10º were observed in the diffraction pattern of CRL-SBA-15. However, same could not be due to the loss of structural order of SBA-15 but caused by the CRL immobilization (Manyar *et al.*, 2008). Thus physical adsorption of lipase does not primarily affect the framework structure of the SBA-15 support material.

The powder XRD pattern of Ti/SiO_2 shows a sharp peak at 0.2° and one broad peak at 1.1° were observed. Upon lipase immobilization the peak at 0.2° shifted at 0.5°, while peaks at 1.1 was no longer found in the XRD patterns of the CRL-Ti/SiO_2. Thus lipase immobilization was found to enhance the amorphous nature of all three mesoporous supports.

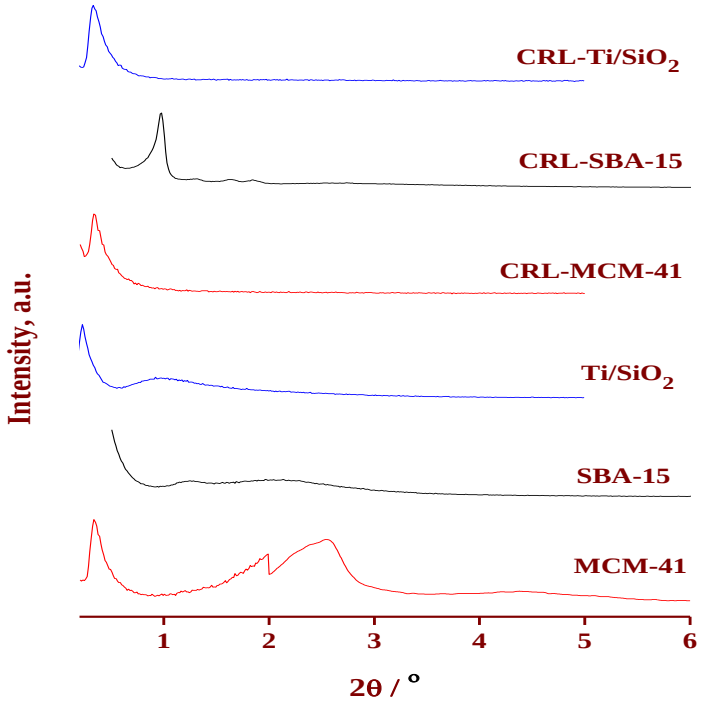

Figure 4.1. Comparison of the XRD patterns of the mesoporous material with lipase immobilized one.

4.3.1.2 Scanning and Transmission Electron Microscopy Study

To study the surface morphology and particle size, SEM images of pure and lipase immobilized mesoporous materials were recorded and compared in Figure 4.2. Pure MCM-41, was found to exist in the form of particles of irregular geometries (Figure 4.2a). The average particle size was found to be less than one micron. Similar type of SEM image (Figure 4.2b) was observed for CRL-MCM-41, to support that lipase immobilization doesn't have any significant effect on the surface morphology or macrostructure of MCM-41 particles. The SEM images of SBA-15 and Ti/SiO_2 (Figure 4.2c and 4.2e) indicated that these have formed as irregular shaped particle of ~ 5μm size. The lipase immobilization was not found to affect the macro structure or size of these particles as revelled by the SEM

images (Figure 4.2d and 4.2f) of CRL-SBA-15 and CRL-Ti/SiO_2. Presences of nitrogen and carbon peaks in the EDX spectrum of CRL-immobilized material indicate the presence of lipase over the mesoporous material.

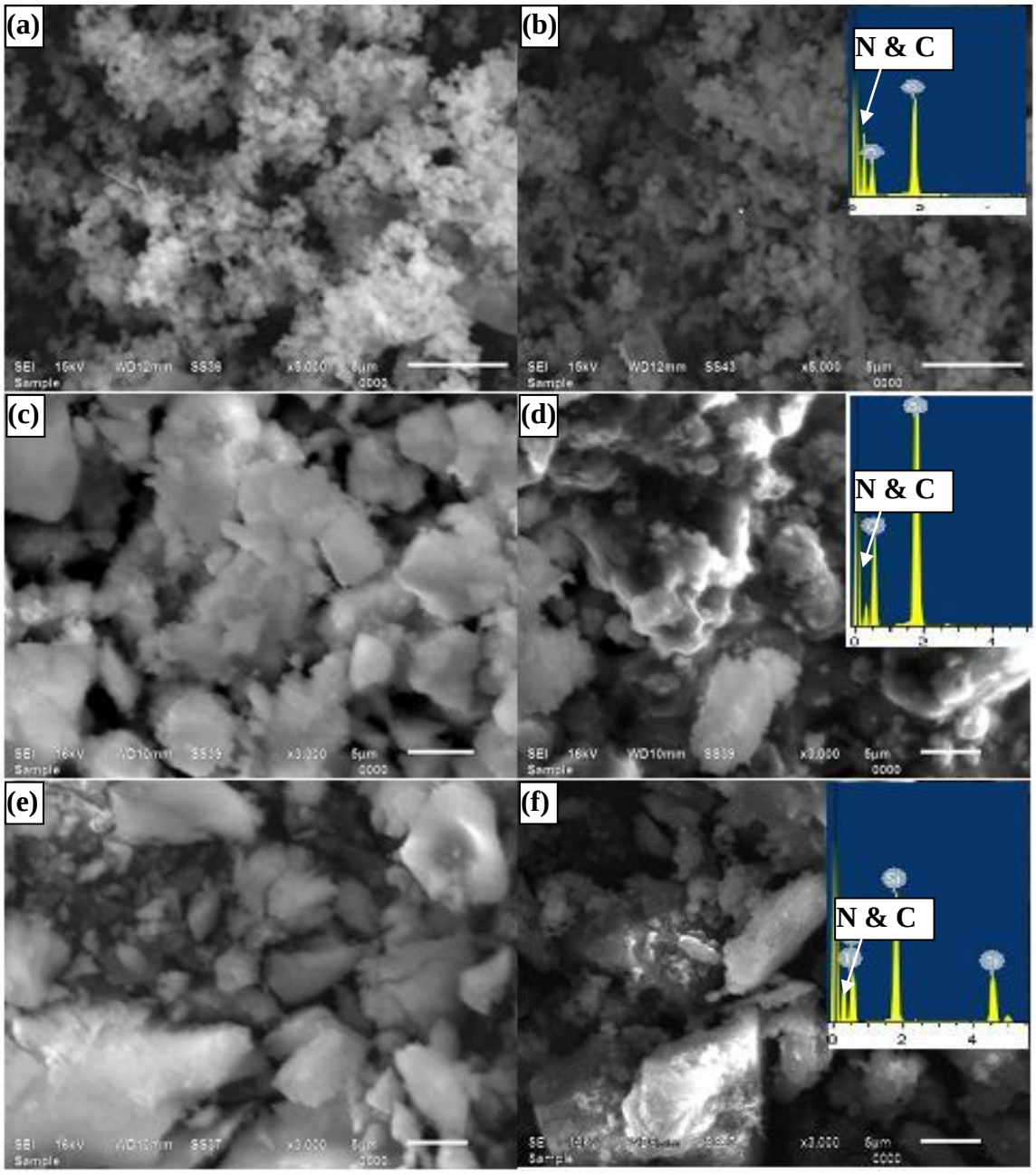

Figure 4.2. SEM images of (a) MCM-41 (b) CRL-MCM-41(c) SBA-15 (d) CRL-SBA-15(e) Ti/SiO_2 and (f) CRL- Ti/SiO_2.

The TEM images have shown the porous structure of MCM-41 and SBA-15. The pore size from the TEM images was observed ~ 8 and 9 nm in case of MCM and SBA materials, respectively. Thus size of the mesoporous material is larger than the molecular size of the lipase protein (4-6 nm). On the other hand Ti/SiO_2 has formed as agglomerated particles and with no visible pores in TEM images. Upon lipase immobilization the support particles show aggregation particles and porous structure of SBA and MCM material no longer remain visible as shown in Figure 4.3b and 4.3d. Agglomeration of the particles is more prominent in case of Ti/SiO_2 upon lipase immobilization (Figure 4.3f).

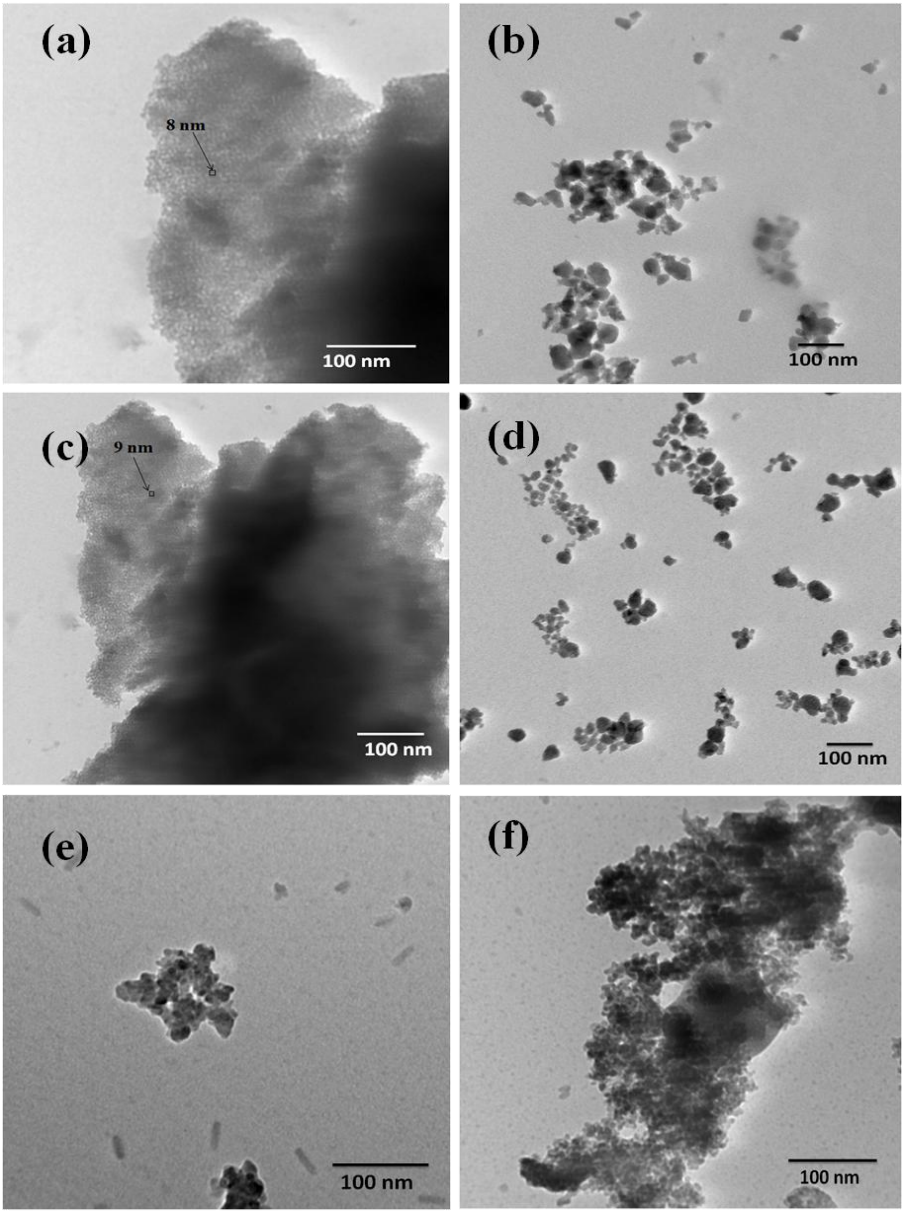

Figure 4.3. TEM images of (a) MCM-41 (b) CRL-MCM-41(c) SBA-15 (d) CRL-SBA-15(e) Ti/SiO_2 and (f) CRL-Ti/SiO_2.

4.3.1.3 FT-IR studies

In order to show the adsorption of lipase on MCM-41, SBA-15 and Ti/SiO_2, the FT-IR spectrum of support, native lipase and lipase supported mesoporous material were compared in Figure 4.4. Pure MCM-41, SBA-15 and Ti/SiO_2 show an intense band at ~1080 cm^{-1} and a medium band at ~803 cm^{-1} due to Si—O asymmetric and symmetric stretching vibrations, respectively (Tan *et al.*, 2010). The amide I band due to C=O stretching and amide II band due to N—H bending vibrations in native lipase appeared at 1660 and 1562 cm^{-1}, respectively. In case of immobilized enzyme, a shift in amide I and II bands were observed as they appeared at ~1645 and ~1540 cm^{-1}, respectively. The small shift in the amide bands of lipase upon immobilization is consistent with the physical adsorption of the enzyme on the matrix.

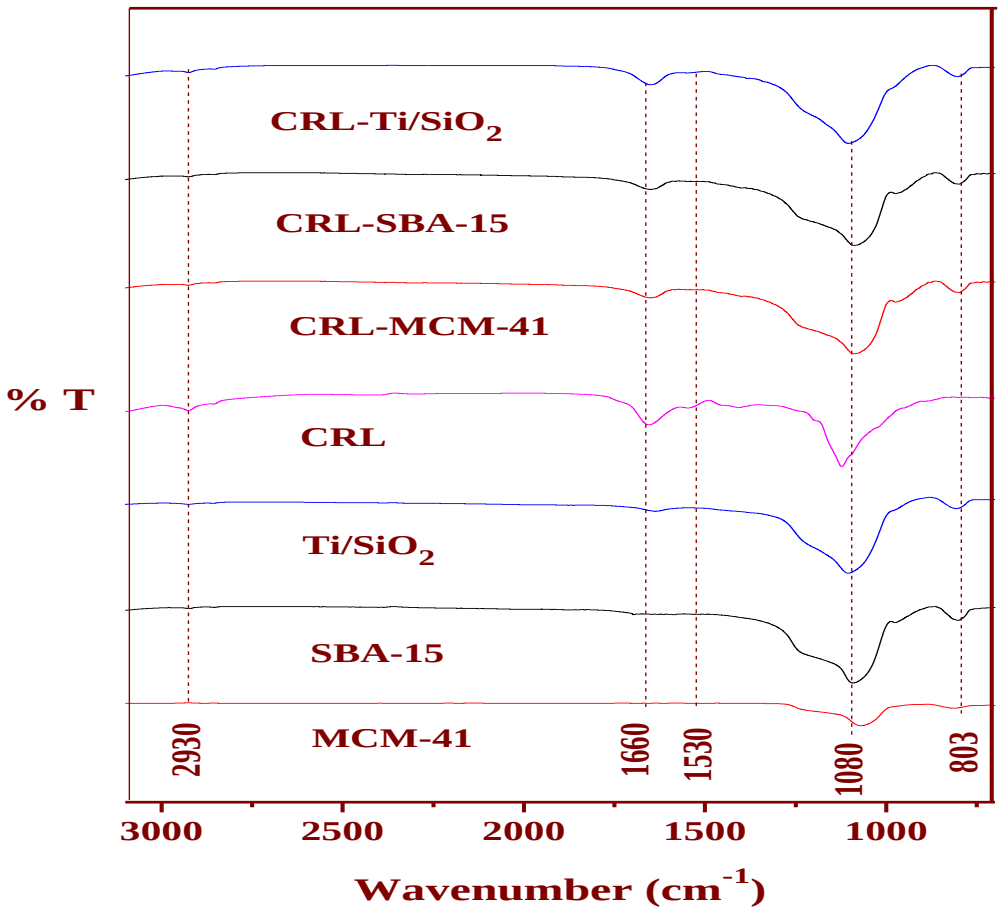

Figure 4.4. Comparison of FT-IR spectra of support material, native-CRL and lipase immobilized material.

The band observed at ~ 2900-2930 cm^{-1}, in case of native and immobilized lipase was due to the –CH, $–CH_2$ and $–CH_3$, groups of the amide chain (Natalello *et al*., 2005). The bands corresponding to Si—O vibrations were also observed in the FTIR spectra of lipase supported mesoporous material to support the immobilization of CRL over mesoporous material.

4.3.2 Parameters effecting lipase immobilization

4.3.2.1 Effect of pH on lipase immobilization on MCM-41

To study the effect of pH on immobilization, enzyme solutions of 3.4 mg/mL concentration were prepared in the buffer solutions of 3-8 pHs. This enzyme solution (20 ml) was agitated with 80 mg MCM-41 at a speed of 160 shakes/min at 20 ºC. The forces involved between lipase and MCM-41, during immobilization, could be electrostatic interactions, hydrogen bonding, weak Van der Waals interactions, and hydrophobic interactions (Tan *et al*., 2010). Out of these, electrostatic force plays an important role during physical adsorption of enzyme on MCM support. As could be seen in Figure 4.5, the maximum enzyme adsorption (250 mg/g) on support was observed at pH 6 (after 4 h) may be due to the maximum electrostatic force of attraction between CRL and MCM support at this pH. On increasing the pH > 6, the surface of CRL will gradually acquire more negative charge. This will result more repulsion between enzyme and support, and could be the reason for the lesser adsorption of enzyme on MCM support at pH > 6.

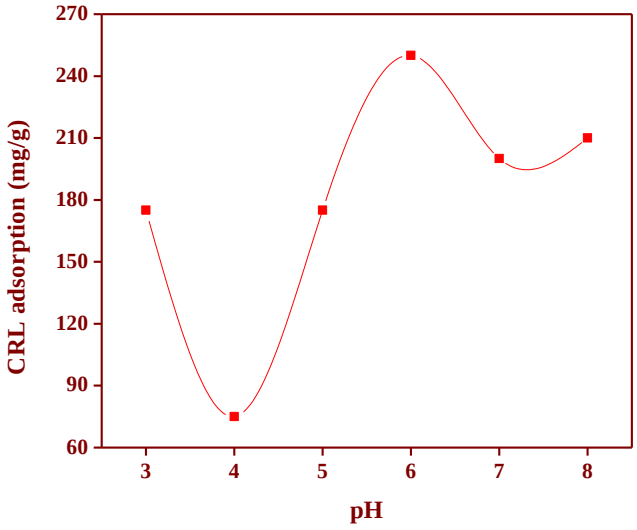

Figure 4.5. Effect of pH on physical adsorption of CRL on MCM-41

4.3.2.2 Effect of contact time on lipase immobilization on MCM-41

In order to determine the optimum contact time between CRL and MCM-41 to achieve the maximum lipase adsorption, 20 ml CRL solution of 3.4 mg/ml concentration (pH = 6) was shaken with 80 mg MCM-41 at a speed of 160 shakes/min at 20 ºC. The amount of immobilized lipase was determined by recording the absorption spectra of supernatant after every two hours at 257 nm and following the literature reported procedure (Tan *et al*., 2010). As could be seen in Figure 4.6, the maximum enzyme adsorption of 250 mg/g was observed after 4 h of contact time. On increasing the contact time beyond 4 h, the amount of adsorbed enzyme was found to decrease gradually due to the partial leaching of the enzyme back into the solution.

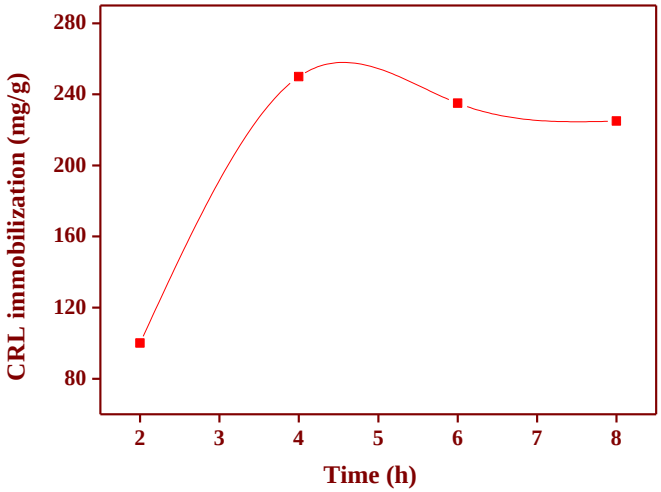

Figure 4.6. Effect of time on immobilization of CRL on MCM-41

Similarly the optimum conditions for the lipase immobilization over SBA-15 and Ti/SiO_2 was established and compared in table 4.1.

Table 4.1. Optimized parameters for the maximum lipase immobilization, by physical adsorption method, on MCM-41, SBA-15 and Ti/SiO_2.

Parameters/support→ ↓	**MCM-41**	**SBA-15**	**Ti/SiO_2**
pH	6	3	6
Time (h)	4	2	6
Enzyme immobilized (mg/g)	250	300	150

4.3.3 Optimization of CRL-MCM-41 catalyzed transesterification of cotton seed oil

Transesterification reaction of cotton seed oil with methanol was performed in presence of all three solid biocatalysts to obtain their optimum activity. In this section the activity of CRL-MCM-41 has been discussed in detail.

4.3.3.1 Effect of pH of reaction medium

The CRL-MCM-41 was used as solid biocatalyst to carry out the transesterification of cotton seed oil. In order to determine the optimum pH to achieve the maximum activity of immobilized lipase, the transesterification reactions were performed in the pH range of 6-9. The optimization of pH is important as it not only influences the lipase activity but also yield of transesterified product. To study the affect of pH, transesterification reaction of methanol with cotton seed oil (12:1 molar ratio) has been performed in the presence of 5 wt% CRL-MCM-41 (with respect to oil) for 48 h at 40 ºC. The pH of the reaction mixture has been maintained by adding the 2 mL buffer solution of desired pH.

The maximum FAMEs yield (98%) was achieved when reaction was performed at pH 8, as shown in Figure 4.7. The FAMEs yields were found to reduce when the same reaction was performed at pH other than 8, due to the lesser activity of CRL-MCM-41 at these pHs.

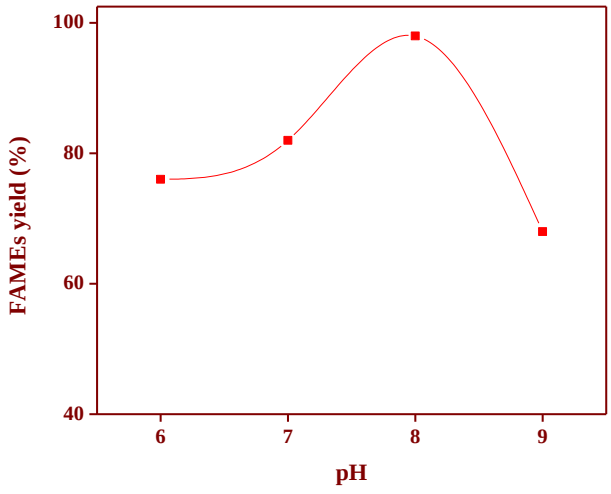

Figure 4.7. Effect of pH on CRL-MCM-41 catalyzed transesterification of cotton seed oil with MeOH. Reaction conditions: alcohol/oil molar ratio = 12:1, enzyme = 5 wt% of oil, stirring speed = 250 rpm at 40 ºC.

4.3.3.2 Effect of temperature

Selection of the appropriate temperature for any enzyme catalyzed reaction is extremely important as most of the enzyme show optimum activity at a particular temperature. In order to determine the optimum temperature for the CRL-MCM-41, the transesterification reaction of cotton seed oil with methanol was performed in the temperature range of 20-50 ºC at pH = 8. As evident from Figure 4.8, the FAMEs yield was found to increase gradually as the reaction temperature was increased from 20-40 ºC, and a maximum (98%) FAMEs yield were obtained at 40 ºC. A further increase in temperature (> 40 ºC) was found to reduce the FAMEs yield, and only 50% conversion was achieved at 60 ºC. This could be due to the partial denaturation of enzyme at higher temperatures which results in lesser enzyme activity.

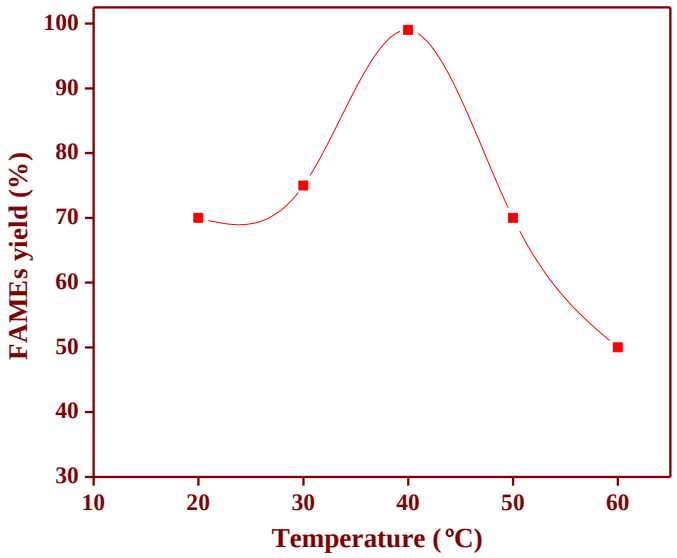

Figure 4.8. Effect of temperature on CRL-MCM-41 catalyzed transesterification of cotton seed oil with MeOH. Reaction conditions: alcohol/oil molar ratio = 12:1, enzyme = 5 wt% of oil, buffer solution = 2 mL (pH = 8) and stirring speed = 250 rpm.

4.3.3.3 Effect of Methanol/oil molar ratio

The minimum theoretical methanol to oil molar ratio required for the complete transesterification of oil to biodiesel is 3:1. Transesterification reactions, being reversible, usually are performed in the presence of excess methanol concentration. However, in case of lipase catalyzed transesterification, use of higher methanol concentration was not encouraged as it is not only toxic but also deactivate the enzyme (Shimada *et al*., 2002). In

order to determine the optimum methanol to oil molar ratio, the transesterification reactions were performed using 5 wt% (with respect to oil) CRL-MCM-41 at pH 8 and 40 ºC, and varying the methanol to oil molar ratio in the range of 3:1 to 15:1. In order to prevent the exposure of enzyme to higher methanol concentration, later was added in three equally divided amounts after every 4 h of reaction duration. The FAMEs yield was found to increase as methanol to oil ratio was increased from 3:1 to 12:1 as shown in Figure 4.9. A 98% conversion of oil to FAMEs was achieved when 12:1 molar ratio of methanol to oil was used. A further increase in methanol to oil molar ratio was found to reduce the FAMEs yield and at 15:1 molar ratio only 65% conversion was achieved. This could be due to the partial deactivation of the immobilized enzyme in presence of higher methanol concentration.

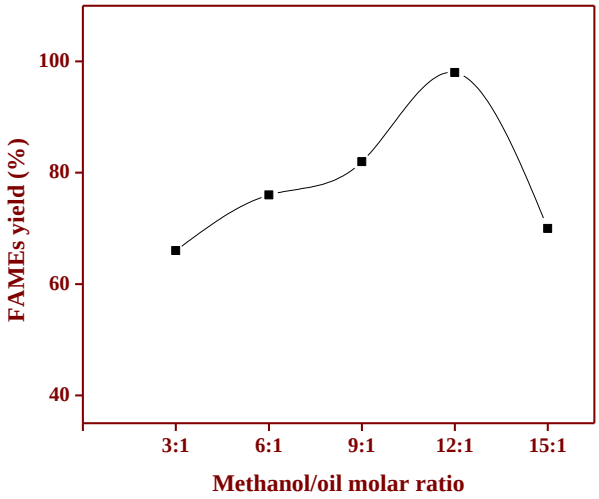

Figure 4.9. Effect of methanol/oil molar ratio on CRL-MCM-41 catalyzed transesterification of cotton seed oil. Reaction conditions: CRL-MCM-41= 5 wt% of oil, buffer solution = 2 mL (pH = 8) and stirring speed = 250 rpm at 40 ºC.

Hence, under the optimized reaction conditions, *viz.*, methanol to cotton seed oil molar ratio of 12:1, 5 wt% CRL-MCM-41 (with respect to oil) at pH 8, 40 ºC and 48 h of reaction duration, a 98% conversion of cotton seed oil in to corresponding FAMEs was achieved. The reactions parameters for the transesterification reactions catalyzed by CRL-SBA-15 and CRL-Ti/SiO_2, were also optimized and given in Table 4.2.

Table 4.2. Optimized reaction conditions for the transesterification reactions catalyzed by CRL-MCM-41, CRL-SBA-15 and CRL-Ti/SiO_2.

Reaction parameters	**CRL-MCM-41**	**CRL-SBA-15**	**CRL-Ti/SiO_2**
Biocatalyst amount (wt% of oil)	5	5	5
Methanol/oil (m/m)	12:1	12:1	12:1
Reaction time (h)	48	68	72
Reaction temperature (^oC)	40	40	40
FAMEs yield (%)	98	98	74

Thus on the basis of reaction parameters CRL-MCM-41 was found to be most active while CRL-Ti/SiO_2 least active as it yielded only partial conversion of CSO even after prolonged reaction duration of 74 h.

4.3.4 Reusability of immobilized enzyme

In order to test the reusability of the immobilized enzymes, they were separated from the reaction mixture by centrifugation and washed successively with methanol and hexane to remove any adsorbed reagents from their surfaces. The regenerated solid biocatalysts were employed for the transesterification of cotton seed oil under the same experimental condition and regeneration methods. As could be seen from Figure 4.10, CRL-MCM-41 and CRL-SBA-15 were found to loss the activity in second catalytic run. The significant drop in activity of solid biocatalyst may be either due to the leaching of the enzyme from the matrix or due to the partial denaturation of the enzyme while catalyzing the first catalytic cycle.

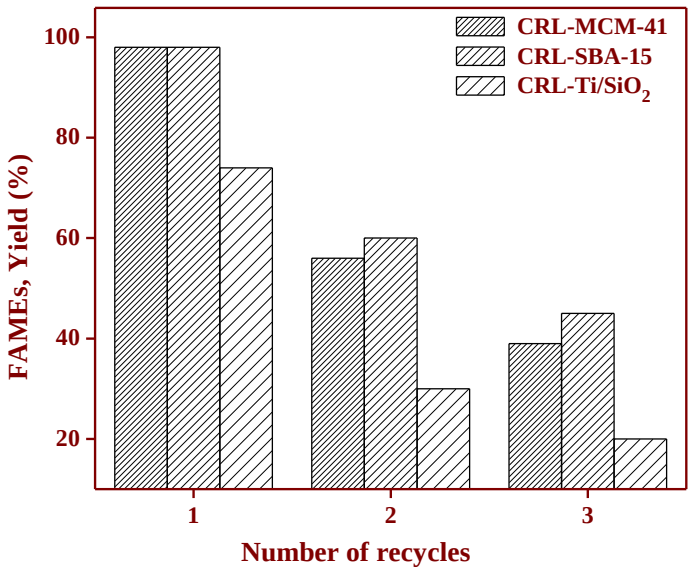

Figure 4.10. Reusability of the CRL-MCM-41, CRL-SBA-15 and CRL-Ti/SiO_2 for the transesterification of CSO.

Leaching of the enzyme form the matrix, while catalyzing the reaction, is a common problem associated with the physically adsorbed enzymes (Park *et al.*, 2009). In order to quantify the extent of leaching, 50 mg of CRL-SBA-15 was mixed with 10 mL of 25 mM buffer solution (pH 3) and stirred (300 rpm) at 20 ºC. After 1 h, the suspension was centrifuged for 20 min at 8000 rpm and supernatant was decanted. The presence of enzyme in supernatant was analyzed by recording absorbance at 257 nm, and absence of any significant absorbance peak at this position (Figure 4.11), supported that enzyme was rarely leached from the matrix.

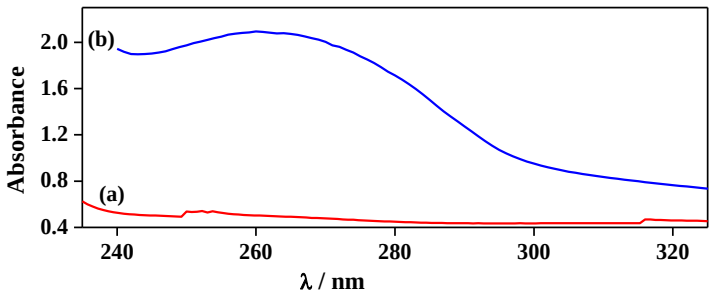

Figure 4.11 - UV-Vis spectra of (a) supernatant obtained from the CRL-SBA-15 and (b) native CRL (3.0 mg mL^{-1}) solution.

Hence, the reduction in enzymatic activity could be due to the partial deactivation of the immobilized enzyme due to presence of methanol and glycerol while catalyzing the first catalytic run (Du *et al*., 2004). At present efforts are in progress, in our lab, to improve the reusability of the immobilized enzyme.

4.3.5 Kinetic study

Theoretically one triglycerides molecule required three molecules of methanol for the complete transesterification, and hence same reaction should follow fourth order kinetic model. However, usually such reactions were performed in presence of excess methanol and hence, pseudo-first order kinetic model is followed (Song *et al*., 2011; Kumar and Ali, 2012). In present study the CRL-MCM-41, CRL-SBA-15 and CRL-Ti/SiO_2 catalyzed transesterification reactions were carried out at five different reaction temperatures *viz.*, 20, 30, 35, 40 and 50 ºC and FAMEs yield obtained at different time intervals were quantified. The values of (pseudo) first order rate constants (k) were calculated from $-\ln(1-X_{me})$ *vs* time plot as shown in Figure 4.12. Complete details of the method and related equations were discussed in detail in chapter 3.

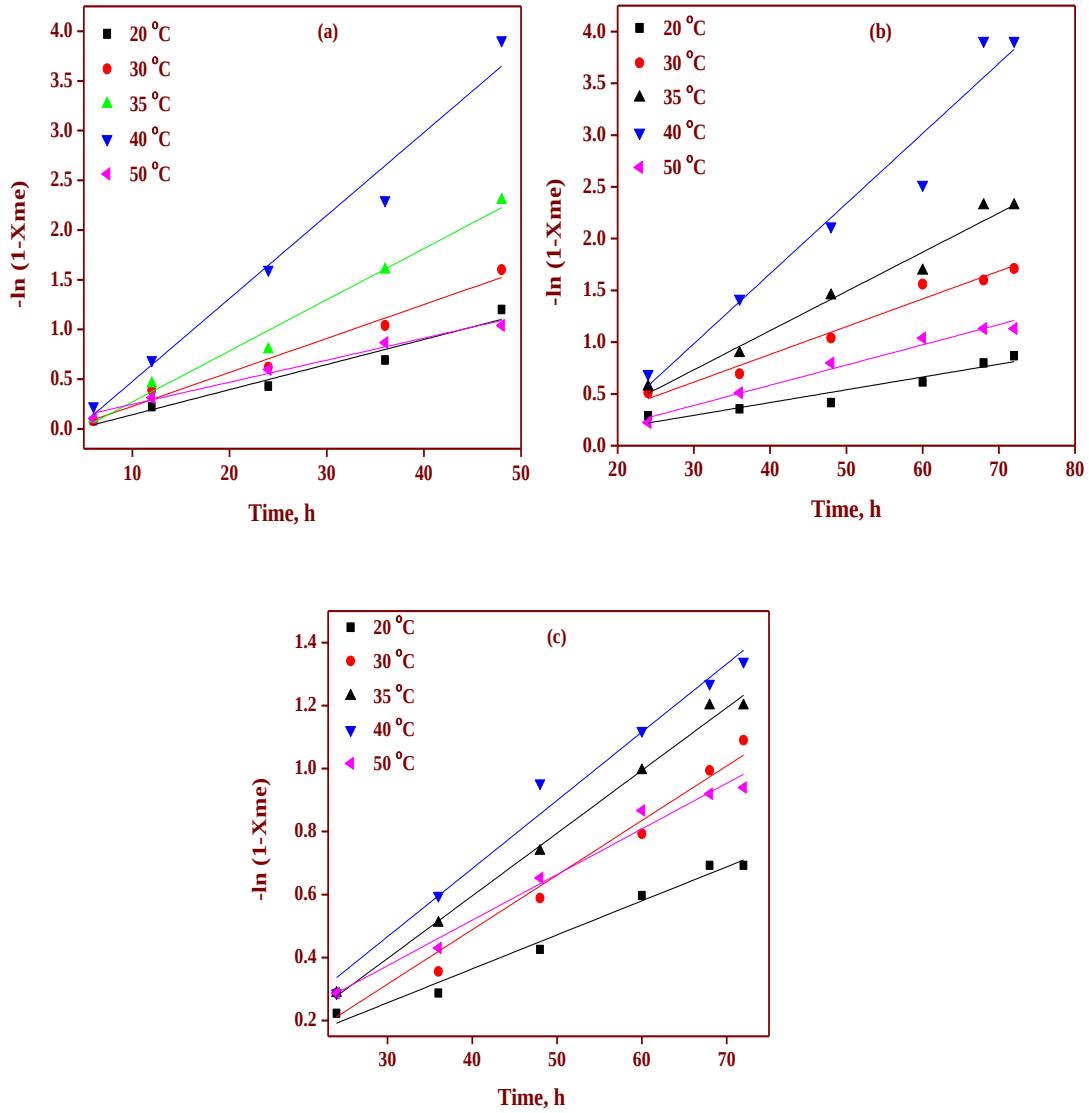

Figure 4.12. Plot of –ln (1-X_{me}) against time (t) at different reaction temperatures; (a) CRL-MCM-41 (b) CRL-SBA-15 and (c) CRL-Ti/SiO_2 catalyzed transesterification reactions, respectively.

The activation energy (E_a) and pre–exponential factor (A) was calculated from the Arrhenius equation given in chapter 3. The E_a and A were calculated from the slope and intercept of 1/T *vs* lnk plot (Figure 4.13), respectively.

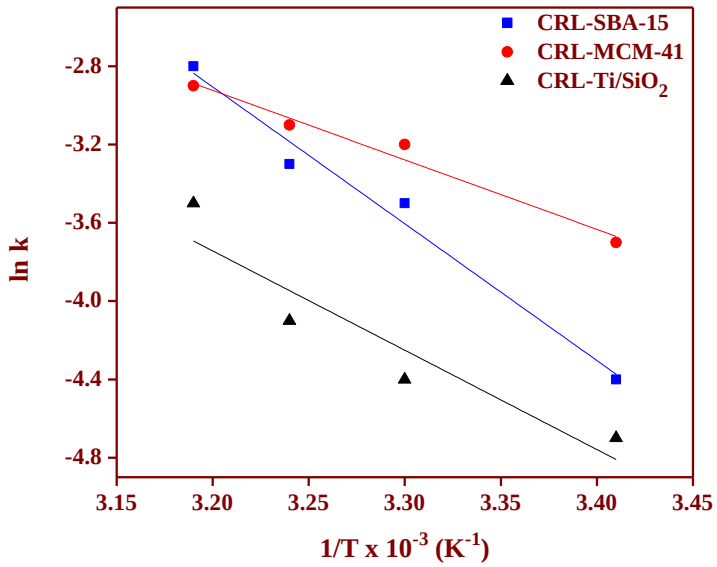

Figure 4.13. Arrhenius plot for the CRL-MCM-41, CRL-SBA-15 and CRL-Ti/SiO_2 catalyzed transesterification of cotton seed oil.

The comparison of rate constant, activation energy and pre–exponential factor is given in Table 4.3 and minimum activation energy of 7.05 kcal mol^{-1} was observed in case of CRL-MCM-41 catalyzed reaction. The observed values of E_a were found within the range of literature reported values (5-15 kcal mol^{-1}) for the lipase catalyzed transesterification reactions (Pogaku *et al.*, 2012).

Table 4.3. Comparison of the rate constant (k), activation energies (E_a) and pre-exponential factor (A) for the CRL-MCM-41, CRL-SBA-15 and CRL-Ti/SiO_2 catalyzed transesterification.

Parameters	**CRL-MCM-41**	**CRL-SBA-15**	**CRL-Ti/SiO_2**
Rate constant (k) h^{-1}	0.083	0.067	0.021
Activation energy (Ea) kcal mol^{-1}	7.05	13.86	10.07
Pre-exponential factor (A) min^{-1}	4.57 x 10^3	2.75 x 10^8	2.63 x 10^5

4.4 Conclusions

Present study demonstrated the preparation of MCM-41, SBA-15 and Ti/SiO_2 and their application for the immobilization of *Candida rugosa* lipase by following the physical adsorption technique. The immobilized enzyme was employed as biocatalyst for the transesterification of the methanol with cotton seed oil. The enzyme activity was found to be affected by the pH of reaction medium, reaction temperature and methanol/oil molar ratio. The maximum activity was demonstrated by CRL-MCM-41 which yielded 98% FAMEs conversion using 12:1, methanol to oil molar ratio at 40 ºC in 48 h of reaction period. The physically adsorbed enzyme has demonstrated poor reusability and recovered immobilized enzyme could not able to complete the reaction in second catalytic run.

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Chapter-5

One pot entrapment of lipase within silica particles having oleic acid core: kinetics of transesterification, enzyme stability and reusability studies

Abstract: In order to enhance the reusability, *Rhizomucor miehei* lipase was entrapped in a single step within silica particles having oleic acid core (RML@SiO_2). Characterization of RML@SiO_2 by powder X-ray diffraction, scanning and transmission electron microscopy and Fourier transform infrared studies supported the lipase immobilization within silica particles. The immobilized enzyme was employed for the transesterification of the cotton seed oil with methanol and ethanol. Under the optimum reaction condition of methanol to oil molar ratio of 12:1, stirring speed of 250 rounds/min at 40 ºC, and 5 wt% biocatalyst (with respect to oil), complete transesterification (> 98%) was achieved in 16 h of reaction duration. The immobilized enzyme didn't require any buffer solution or organic solvent for optimum activity, and hence biodiesel and glycerol thus produced was free from any metal ion and organic molecule contamination. The activation energy for the immobilized enzyme catalyzed ethanolysis and methanolysis were found to be 8.36 and 4.72 kcal mol^{-1}, respectively. The immobilized enzyme was recovered from reaction mixture and reused in twelve successive runs without significant loss in activity. Immobilization process not only resulted better reusability but also better stability of the enzyme as no significant loss in activity was observed even storing RML@SiO_2 at room temperature (25-30 °C) over a period of eight months.

Keywords: Immobilization. *Rhizomucor miehei* lipase. Kinetics. Reusability. Enzyme stability. Biodiesel.

5.1 Introduction

In chapter 4, mesoporous material has been employed for the immobilization of the lipase by physical adsorption technique. Although immobilized enzyme was recovered after the reaction, but it demonstrated poor reusability. In literature, besides physical adsorption, encapsulation, entrapment, cross linking and chemical bonding with the functional group anchored on carrier, were also reported (Fukuda *et al.*, 2001; Ali *et al.*, 2011). Physically adsorbed enzyme may leach out easily while catalyzing the reaction, while chemical methods of immobilization although provide better stability, but required complicated derivatization process. Moreover, often enzyme activity was found to be effected adversely due to the conformation changes occurred in enzyme structure as a consequence of chemical bonding between enzyme and carrier. Sol-gel method of enzyme entrapment

provides a better alternative for the lipase immobilization as in this technique enzyme is directly entrapped in a single step within the carrier. This method is not only simple but also often imparts better reusability, activity and stability to the enzyme towards pH, heat and reactant/product molecules as enzyme is partially protected by the carrier (Palla *et al.,* 2011; Souza *et al.*, 2012). Moreover, in order to tune the hydrophilic/hydrophobic environment of the matrix, triglycerides or fatty acids could be added during the immobilization step to achieve optimum lipase activity (Galarneau *et al.*, 2006). Silica due to its inert and non toxic nature is one of the most frequently employed carriers for the lipase entrapment in literature (Hartmann and Jung, 2010).

To further improve the stability and reusability of the immobilized enzyme, in present chapter, solid biocatalyst was prepared by entrapping *Rhizomucor miehei* lipase within silica particles having oleic acid core (RML@SiO_2). The reaction parameters for the RML@SiO_2 catalyzed transesterification of cotton seed oil with methanol and ethanol have been optimized to study the kinetics of the reaction. The prepared solid biocatalyst was reused more than 10 times without significant loss in activity. Further, immobilized enzyme was found to be stable over a period of 8 months even when stored at room temperature.

5.2 Experimental

5.2.1 Immobilization of lipase

Rhizomucor miehei Lipase (RML) was immobilized within silica nanoparticles by entrapment method following the literature reported protocol (Kuwahara *et al.*, 2012) with slight modifications. In a typical experiment, 0.64 ml oleic acid was sonicated with 120 mL deionized water for 10 min to obtain an emulsion and to this 8 mL RML solution (1 wt%) was added. To this, 3 mL TEOS and 0.46 ml APTES was added drop wise and resulting mixture was stirred for 20 min at 30 °C, and left to age for 3 h at the same temperature under static conditions. At this stage a thick gummy material was obtained which was dried at 40 °C for 24 h to achieve solid biocatalyst (RML@SiO_2). The solid biocatalyst, thus obtained, was washed thrice with deionized water, centrifuged at 8000 rpm and finally dried at 40 °C for 48 h. Similar experimental procedure was employed to prepare the *Candida rugosa* and *Aspergillus niger* lipase entrapped silica particles (CRL@SiO_2 and ANL@SiO_2, respectively). The prepared samples were stored in airtight glass sample bottles at room temperature (25-30 °C) and used as and when required.

In order to perform the blank experiments, oleic acid@SiO_2 (without lipase) was also prepared using the above mention method but without adding lipase. To obtain silica particles without enzyme, oleic acid@SiO_2 was calcined at 400 °C for 5 h.

5.2.2 Transesterification activity of immobilized enzymes

To screen the activity of RML@SiO_2 towards the transesterification reactions cotton seed oil was employed as substrate. Prior to the reaction, 0.5 g RML@SiO_2 was activated by soaking in 2 mL deionized water for 15 min. In a typical transesterification reaction a two neck, 25 mL round bottom flask equipped with a water bath, magnetic stirrer and condenser was charged with 0.5 g activated RML@SiO_2, 12 ml CSO and 5.5 ml methanol (12:1 methanol to oil molar ratio). Methanol was added in the reaction mixture in three equally divided amounts at 0 h, 4 h and 8 h of the reaction in order to prevent the exposure of lipase to high methanol concentration. The reaction mixture was magnetically stirred (250 rounds/min) for desired duration at desired temperature. To monitor the progress of the reaction, 0.5 mL of the reaction mixture was withdrawn after every 4 h and subjected to the proton NMR analysis. After the completion of the reaction, the solid enzyme was recovered from the reaction mixture by centrifugation and liquid phase was kept in a separating funnel to separate the lower glycerol layer from the upper FAME layer. Excess alcohol from upper layer was rotary-evaporated to obtain pure FAME. Similar method was also employed for the ethanolysis of cotton seed oil but using ethanol in place of methanol.

5.2.3 FAME and FAEE quantification

Proton NMR technique being simple, non destructive and not requiring any derivatization step was employed for quantification of fatty acid ethyl or methyl esters. Detail description of the method is provided in chapter 2.

Cotton seed oil derived FAMEs **^{1}H-NMR** ($CDCl_3$, δ ppm): 5.3 (m, -C**H**=C**H**-), 3.6 (s, -OC**H_3**), 2.7 (m, -CH=CH-C**H_2**-CH=CH-), 2.3 (m, -C**H_2**-CO-), 2.0 (m, -C**H_2**-CH=CH-), 1.6-1.25 (m, -(C**H_2**)$_n$-), 0.95 (m, -CH=CH-C**H_3**), 0.87 (m, -CH_2-C**H_3**). **^{13}C-NMR** ($CDCl_3$, δ ppm): 174.1 (-**C**O-CH_2-), 129.9 (-**C**H=**C**H-), 77.1 ($CDCl_3$), 51.2 (-O**C**H_3), 34.1 (-CO-**C**H_2-), 31.9 (ω3 -**C**H_2-), 29.66-29.08 (-CH=CH-**C**H_2-, -**C**H_2-), 27.2 (-CH=CH-**C**H_2-CH=CH-), 25.6-24.80 (-CO-CH_2-**C**H_2-), 22.70, 22.47 (ω2 -**C**H_2-) and 14.16 (ω1 -**C**H_3).

Cotton seed oil derived FAEEs **^{1}H-NMR** ($CDCl_3$, δ ppm): 5.3 (m, -C**H**=C**H**-), 4.2 (q, -OC**H_2**-C**H_3**), 2.7 (m, -CH=CH-C**H_2**-CH=CH-), 2.3 (m, -C**H_2**-CO-), 2.0 (m, -C**H_2**-CH=CH-), 1.6-1.25 (m, -(C**H_2**)$_n$-), 0.95 (m, -CH=CH-C**H_3**), 0.87 (m, -CH_2-C**H_3**). **^{13}C-NMR** ($CDCl_3$, δ ppm): 174.09(-**C**O-CH_2-), 129.9 (-**C**H=**C**H-), 77.1 ($CDCl_3$), 61.5 (-O**C**H_2 -CH_3), 34.1 (-CO-**C**H_2-), 31.9 (ω3 -**C**H_2-), 29.66-29.08 (-CH=CH-**C**H_2-, -**C**H_2-), 27.2 (-CH=CH-**C**H_2-CH=CH-), 25.6-24.80 (-CO-CH_2-**C**H_2-), 22.70, 22.47 (ω2 -**C**H_2-) and 14.16 (ω1 -**C**H_3).

5.3 Results and discussion

5.3.1 Characterization of RML@SiO_2

In literature various techniques (Zhou and Hartmann, 2012) were employed for the lipase immobilization on solid support including physical adsorption, cross linking, and entrapment. In present work enzyme has been entrapped in a single step within the silica particles having oleic acid core. The oleic acid micelles in this technique not only acts as template for the formation of spherical silica particles, but also improves the hydrophilic/hydrophobic nature of the support and didn't allow the shrinkage of silica particles while drying (Rodrigues *et al*., 2010). The enzyme molecules remain entrapped in the porous wall of silica as shown in Figure 5.1. The prepared enzyme has been characterized by powder XRD, SEM, TEM, FTIR and BET-surface area measurement studies.

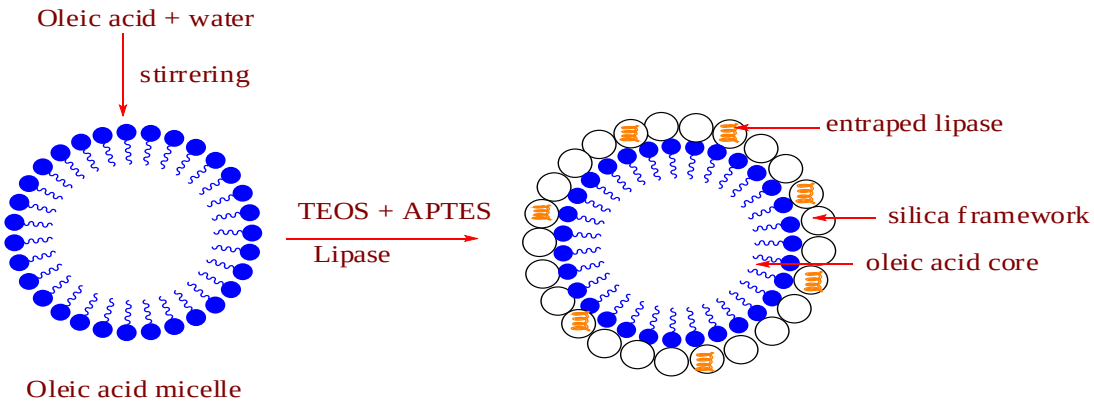

Figure 5.1. Pictorial presentation of the lipase entrapment in silica shell with oleic acid core

The SEM image shown in Figure 5.2a, gives the surface morphology and particle size of RML@SiO_2. As could be seen from the image, RML@SiO_2 consists of

agglomerated spherical silica nanoparticles of ~ 1-2 μm diameters. Presence of nitrogen and carbon has been observed by the Electron Diffraction X-ray analysis (EDX) of RML@SiO_2, to support the immobilization of the lipase within silica particles. High carbon percentage (55.55 wt%) is due to the presence of carbon of oleic acid and lipase enzyme. Analysis of the same particles by TEM Figure 5.2b, reveals that these particles are the clusters of further smaller spherical particles with an average size of ~ 55 nm. The SiO_2 shell wall thickness was observed ~11 nm.

The BET surface area measurement has also supported the occupancy of silica pores by organic molecules. The specific surface area of as synthesized RML@SiO_2 was found to be much smaller (25.76 m^2 g^{-1}), due to the pore plugging of the silica by oleic acid and RML, than the calcined one (319.01 m^2 g^{-1}).

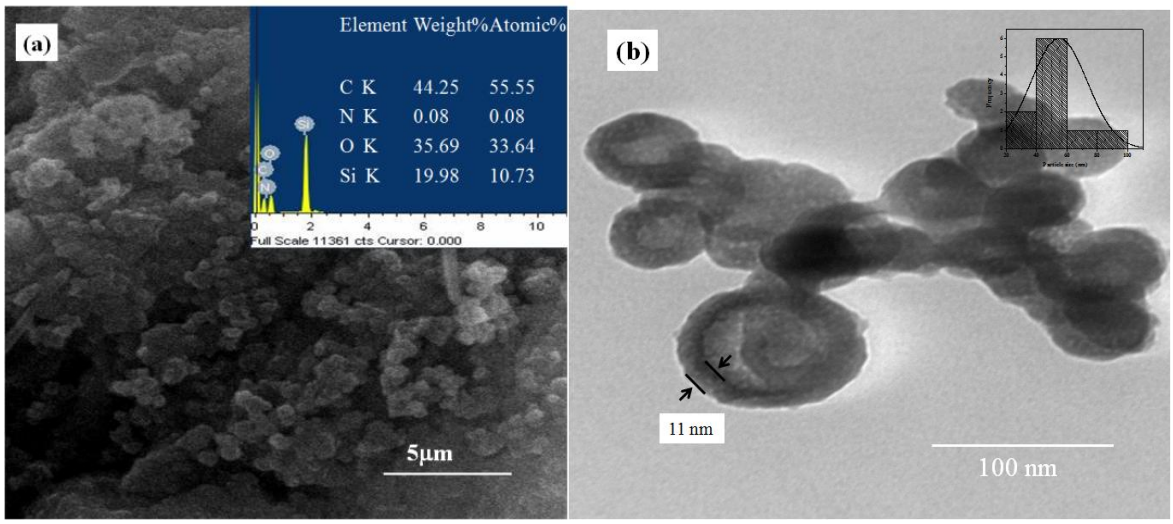

Figure 5.2. (a) SEM, (EDX is shown in inset) and (b) TEM images of RML@SiO_2 (inset shows the particle size distribution)

The XRD patterns of as-synthesized and calcined RML@SiO_2 are compared in Figure 5.3. No distinct peak was observed in the diffraction pattern of as synthesized solid biocatalyst, to support its amorphous nature. However, calcination of RML@SiO_2 at 400 °C has initiated some long range arrangements in the silica structure as supported by the appearance of a diffraction peak at $2\theta = 3.9°$, corresponding to the (110) plane of hexagonal silica phase (Li and Zhao, 2013).

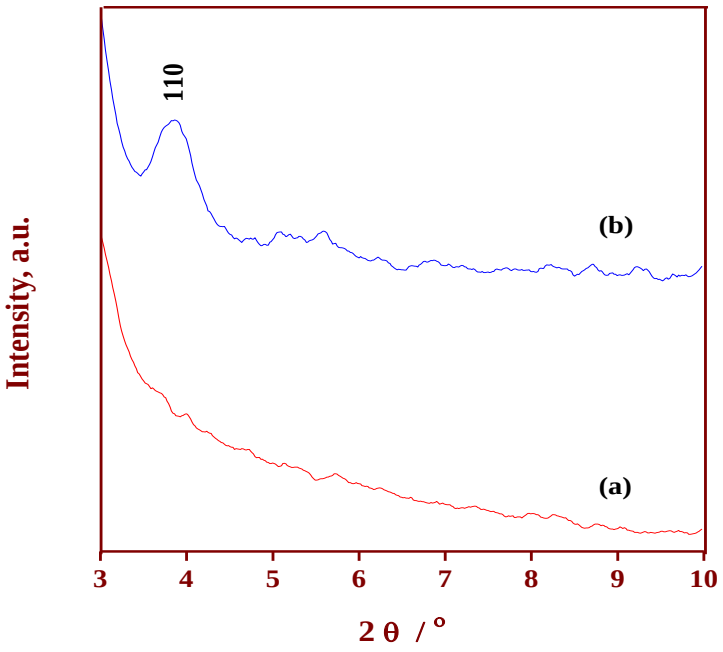

Figure 5.3. Comparison of the XRD patterns of (a) as synthesized one with (b) calcined RML@SiO_2

To further demonstrate that RML@SiO_2 contains lipase as well as oleic acid molecules, FT-IR spectrum of pure silica, RML@SiO_2 without oleic acid, oleic acid@SiO_2 and RML@SiO_2 have been compared in Figure 5.4. Characteristic bands at 1080 and 803cm^{-1} due to Si-O asymmetric and symmetric stretching vibrations, respectively, were observed in all the samples due to the presence of silica framework (Figure 5.4a-d). Entrapment of RML within silica frame work was supported by the presence of amide I and amide II bands at ~ 1648 and 1548 cm^{-1}, respectively (Figure 5.4c and d). Oleic acid entrapment in silica particles was supported by the presence of carbonyl band (of carboxylic acid) at ~ 1711 cm^{-1}, in FT-IR spectrum of oleic acid@SiO_2 (Figure 5.4b). Amide as well as carboxylic bands was also observed in RML@SiO_2 to indicate that lipase has been entrapped in silica frame work having oleic acid molecules. Bands observed at ~ 2925 and 2850 cm^{-1} (Figure 5.4b, c and d), due to the symmetric and asymmetric -C-H stretching vibrations, also supported the presence of hydrocarbon groups of oleic acid and lipase molecules.

Upon storage at room temperature the activity of RML@SiO_2 without oleic acid was lost due to the shrinkage of the silica particle. Thus oleic acid is necessary to hold the spherical structure of the silica particles which in turn helps in retaining the activity of the immobilized enzyme.

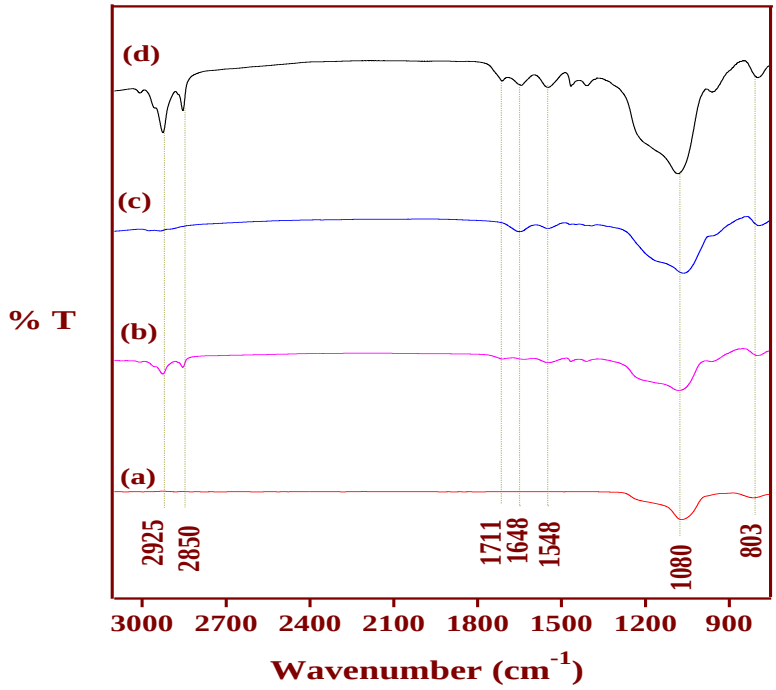

Figure 5.4. Comparison of FT-IR spectra in the range of 800-3000 cm^{-1} (a) silica (b) SiO_2 with oleic acid core (c) RML@SiO_2 without oleic acid and (d) RML@SiO_2 with oleic acid core.

5.3.2 FAME and FAEE characterization

The efficacy of the prepared biocatalyst, RML@SiO_2, was evaluated towards the methanolysis as well ethanolysis of CSO. The ^{1}H and ^{13}C-NMR and GC-MC techniques are employed for the structural elucidation and quantification of the prepared FAME and FAEE molecules. The details of both the methods have already been discussed in chapter 3.

5.3.3 Transesterification activity of RML@SiO_2

5.3.3.1 Effect of buffer, organic solvent, enzyme and support

Most of the literature reported immobilized or native lipases have been employed for the transesterification reaction in presence of buffer solution and/or in organic medium (Kim *et al.*, 2013). Addition of buffer solution of appropriate pH and/or organic solvent during the transesterification reaction although found to enhance the enzyme activity (Huang *et al.*, 2012) but produces the biodiesel and glycerol contaminated with the added molecules. Thus additional purification step is required which not only increases the biodiesel production cost but also generate huge amount of the industrial effluents to cause the environmental pollution. In order to overcome this problem, in present work, deliberately no buffer solution or organic solvent was added during the transesterification reaction.

Initially three different solid biocatalysts were prepared by entrapping CRL, ANL and RML within silica particles having oleic acid core. The methanolysis of CSO performed in presence of CRL@SiO_2 or ANL@SiO_2 yielded only 40 % conversion even after 48 h of reaction duration. Whereas, same reaction when catalyzed by RML@SiO_2 gave > 98% FAME yield. Hence, in present work RML@SiO_2 has been selected for optimizing the reaction parameters. Later under optimized reaction conditions the reusability and stability of RML@SiO_2 has also been evaluated.

In order to study the effect of matrix on transesterification, blank experiments were also carried out by employing either pure SiO_2 or oleic acid@SiO_2 in place of RML@SiO_2. Under such reaction condition negligible conversion (< 5%) of oil to fatty acid alkyl ester was observed. Hence, it would be safe to assume that silica, used as matrix, remain silent during the catalytic process.

5.3.3.2 Effect of alcohol/oil ratio

Theoretically, a minimum of 3:1, alcohol to oil molar ratio is required for the complete transesterification of oil into corresponding fatty acid alkyl esters. However, being a reversible reaction and in order to shift the equilibrium towards forward direction, transesterification is usually performed in presence of excess alcohol. In case of lipase catalyzed reactions use of higher methanol concentration was found to deactivate the

enzyme (Shimada *et al*., 2002). To determine the optimum oil to alcohol molar ratio, transesterification reactions were performed in presence of 5 wt% (with respect to oil) RML@SiO_2 at 40 ºC, and varying the alcohol to oil molar ratio in the range of 3:1 to 18:1. To prevent the exposure of enzyme to high methanol concentration, alcohol was added in three equally divided amounts after every 4 h of reaction duration. The FAME yield was found to increase as methanol to oil ratio was increased from 3:1 to12:1, as shown in Figure 5.5a. A > 98% conversion of oil to FAME was achieved in 16 h of reaction duration when a 12:1 methanol to oil molar ratio was employed. A further increase in methanol concentration ratio was found to reduce the FAME yield, may be due to the partial deactivation of the immobilized enzyme by the high methanol concentration.

At industrial scale biodiesel is usually produced through methanolysis reaction, however, methanol used in this process is a refinery residue and thus, produced biodiesel would not be having 100% renewable carbons. Further, methanol is highly toxic and a non green chemical, hence, there is a need to replace it with non toxic and renewable alcohol.

Ethanol is usually produced from plant sources (e.g. starch, sugar, etc.,) and could be employed as a renewable substitute for methanol in transesterification of vegetable oil. In order to demonstrate the efficacy of the RML@SiO_2 towards ethanolysis, it was also employed for the transesterification of CSO with ethanol as shown in Figure 5.5b. Although, prepared biocatalyst was also found to be effective even towards ethanolysis, however, it required longer duration (24 h) and a higher ethanol to oil molar ratio (15:1) to produce > 98% FAEE yield.

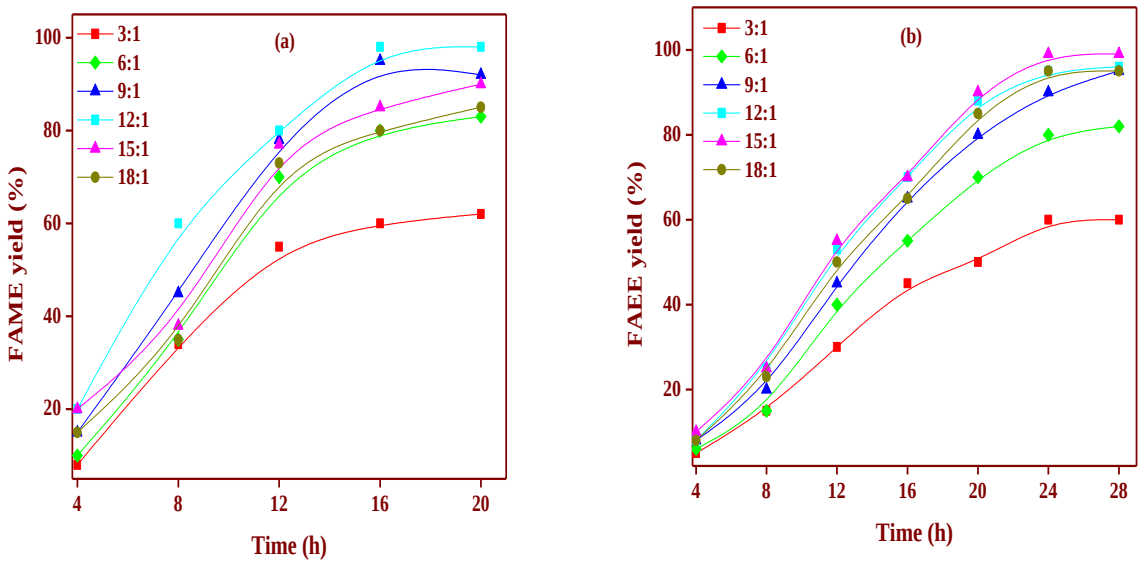

Figure 5.5. Effect of alcohol to oil molar ratio on RML@SiO_2 catalyzed transesterification of CSO at 40 ºC with (a) methanol and (b) ethanol.

5.3.3.3 Effect of temperature

Enzyme activity is sensitive to the temperature and too low or too high temperature could reduce the enzyme activity to a significant extent. Moreover, at relatively high temperature enzyme could be denatured irreversibly and hence, it is vital to know the temperature for the optimum enzyme activity. The transesterification reactions of CSO with methanol (1:12 molar ratio) and ethanol (1:15 molar ratio) were performed in presence of 5 wt% RML@SiO_2 (with respect to oil) by varying the reaction temperature 20-50 ºC. As evident from Figure 5.6a and 5.6b, on increasing the temperature from 20 to 40 ºC, lipase activity was found to increase gradually and a complete conversion was achieved at 40 ºC in 16 and 24 h in case of methanolysis and ethanolysis, respectively. A further increase in reaction temperature (> 40 ºC) resulted in reduced enzymatic activity due to the thermal deactivation of the enzyme.

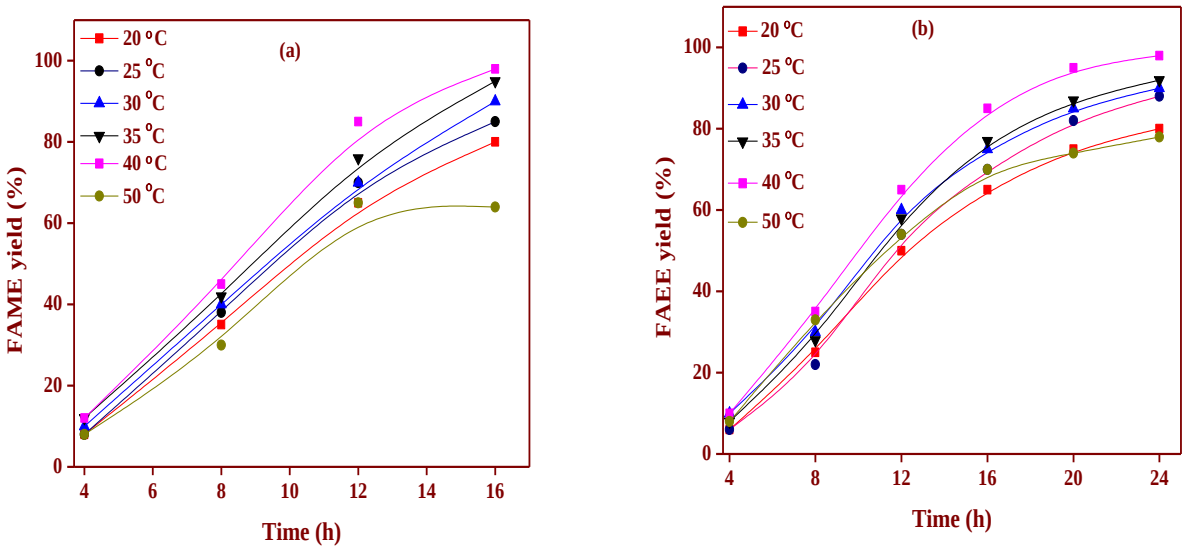

Figure 5.6. Effect of temperature on RML@SiO_2 catalyzed (a) methanolysis and (b) ethanolysis of CSO.

Thus optimized reaction conditions for RML@SiO_2 activity are 12:1 methanol to oil or 15:1 ethanol to oil molar ratio in presence of 5 wt% biocatalyst at 40 ºC reaction temperature and 250 rpm stirring speed.

5.3.4 Effect of FFA and moisture contents on RML@SiO_2 activity

At industrial scale biodiesel is produced by the transesterification of vegetable oils in presence of homogeneous base (*e.g.* NaOH, KOH etc.) catalysts. Presence of FFA (> 0.5 wt%) and moisture (> 0.3 wt%) contents in feedstock leads to the formation of soap in place of biodiesel, when reaction is catalyzed by homogeneous base catalyst (Kumar and Ali, 2012). Hence, high quality costly refined vegetable oils (moisture and FFA free) must be employed as feedstock for the biodiesel production to utilize homogeneous chemical catalysts. In present work water is required for RML@SiO_2 activation and thus presence of moisture (up to 2 wt%) in reaction mixture was not found to effect the enzyme activity adversely.

In order to test the effect of FFA on RML@SiO_2 activity, transesterification of a variety of triglycerides (*viz.*, SO, CSO, UCO, and JO) having up to 6.8 wt% FFA, was performed with methanol under optimized reaction conditions. The complete transesterification of all these oils in respective FAMEs were obtained as given in Figure 5.7. Application of UCO and JO as feedstock in presence of NaOH homogenous catalyst leads to the formation of soap instead of FAME. Thus, prepared biocatalyst is clearly advantageous over its chemical counterpart owing to its greater moisture and FFA tolerance ability.

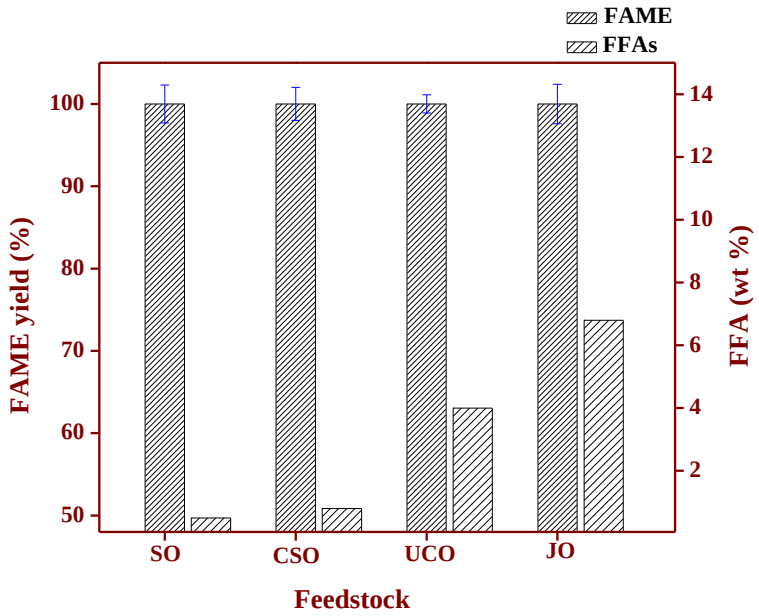

Figure 5.7. Effect of FFA on RML@SiO_2 catalyzed transesterification of various oils. Reaction conditions; MeOH/oil molar ratio = 12:1, biocatalyst = 5 wt% of oil and reactions temperature = 40 ºC.

5.3.5 Recyclability and stability of RML@SiO_2

Extremely high cost of lipase in comparison to chemical catalyst is a major hurdle for commercialization of enzyme catalyzed transesterification reaction. The rationale behind the lipase immobilization includes ease of separation of solid biocatalyst from the reaction mixture and further reusability of the recovered biocatalyst. Repeated use of biocatalyst could make the process economically viable by reducing the effective cost of enzyme. To test the reusability of the recovered RML@SiO_2 it was employed for the methanolysis of CSO under optimized reaction conditions. Upon completion of reaction solid biocatalyst

was recovered from the reaction mixture by centrifugation, and washed successively with hexane and water to remove any adsorbed molecule from its surface. The recovered enzyme was employed in 12 successive runs under the same experimental and regeneration methods. As could be seen from Figure 5.8, no significant loss in the activity of immobilized enzyme was observed and a FAME yield of ~97% was maintained even after 12 cycles.

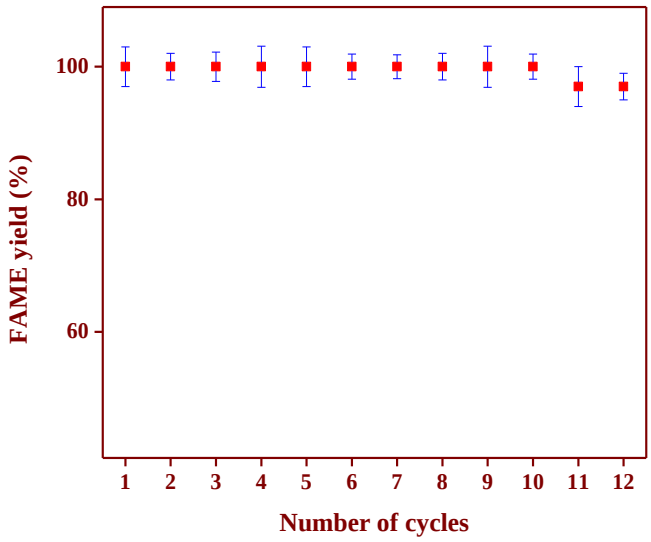

Figure 5.8. Reusability of RML@SiO_2 towards the transesterification of the CSO.

Pure enzymes are temperature sensitive and hence, usually required low temperature for their storage. As per the specifications native RML employed in present work must be stored at 4 ºC. This is another obstacle for the commercialization of the lipase as additional infrastructure would be required to store enzyme for longer duration. In order to study the stability of RML@SiO_2 it was stored at room temperature (25-30 °C) and employed for the transesterification of CSO with methanol over a period of eight months after a time gap of one month. As evident from Figure 5.9, immobilized lipase retains 100% activity even after 6 months of storage at room temperature. After that a fractional loss in activity was observed and ~ 95% activity was retained by RML@SiO_2 even after 8 months of storage. The greater stability of the immobilized enzyme could be due to the stabilization of lipase in its native structural state upon entrapment in silica particles. This indicates that a very stable immobilized enzyme was obtained following the immobilized method discussed in present research work. Meunier and Legge (2012) recently reported the diatomaceous earth

supported lipase with a long self life of ~ 1.5 years, however, immobilized enzyme required low temperature (4 °C) for storage to maintain the activity.

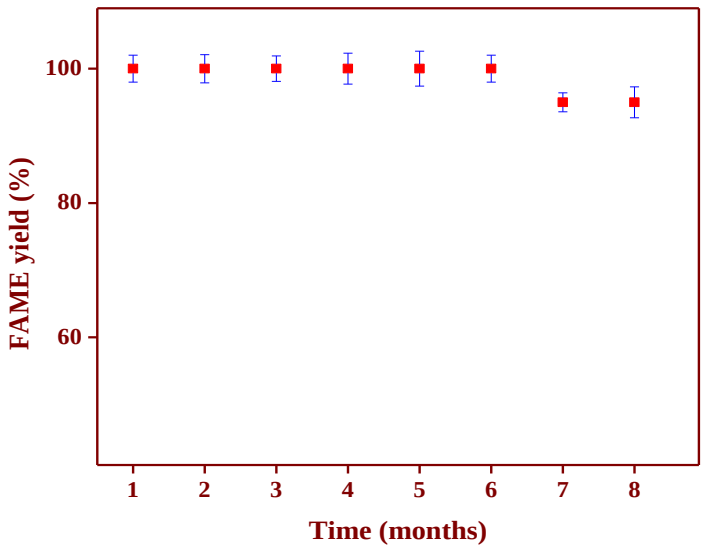

Figure 5.9. Stability test of RML@SiO_2 (stored at 25-30 ºC).

5.3.6 Kinetic study

As described in chapter 3, the pseudo-first order rate law could be employed to study the kinetics of the transesterification of triglycerides. In order to validate the kinetics of the RML@SiO_2 catalyzed methanolysis and ethanolysis reactions were carried out at 20, 25, 30, 35, 40 and 50 ºC reaction temperature. The linear nature of $-\ln(1-X_{me})$ or $-\ln(1-X_{ee})$ *vs* t plots, as shown in Fig. 5.10, supported that RML@SiO_2 catalyzed transesterification reaction has followed pseudo first order kinetic equation.

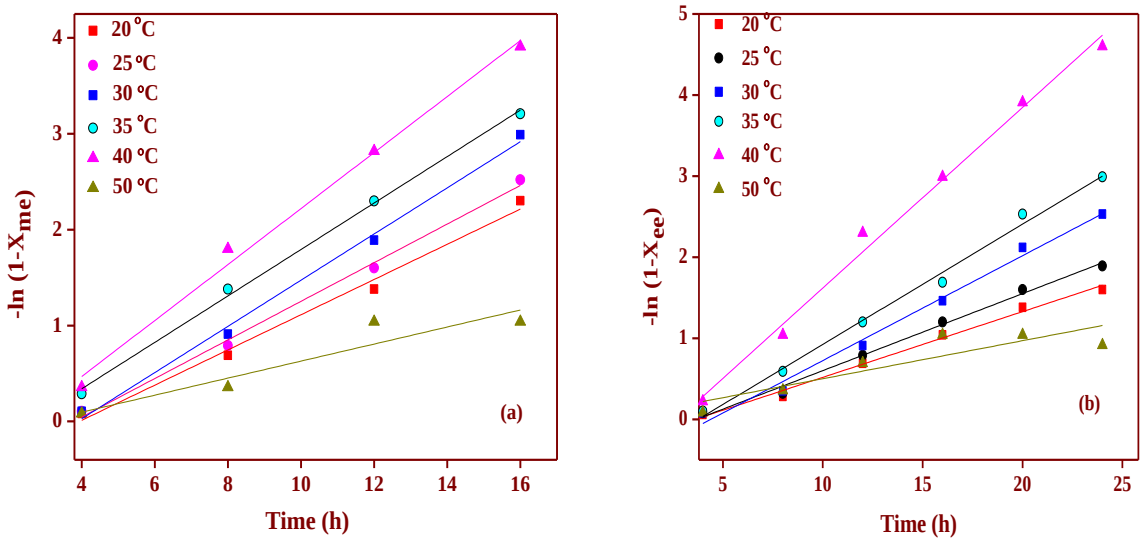

Figure 5.10. Plot of (a) –ln (1-X_{me}) and (b) –ln (1-X_{ee}) against time (t) at different reaction temperatures; where X_{me} and X_{ee}, are the fractions of FAME and FAEE, respectively.

The rate constants (*k*), as calculated from Figure 5.10a and 5.10b, was found to be maximum 4.07 $\times 10^{-3}$ min^{-1} and 2.47 $\times 10^{-3}$ min^{-1} at 40 ºC, in case of methanolysis and ethanolysis, respectively.

Figure 5.11, shows the Arrhenius plot and in this plot the slope and intercept are equals to $-E_a/RT$, and A, respectively. The values of E_a were found to be 4.72 and 8.36 kcal mol^{-1} for the methanolysis and ethanolysis, respectively and values for A, were calculated as 4.89×10^2 and 1.12×10^5 min^{-1} for the methanolysis and ethanolysis, respectively. The observed values of E_a are found within the range of literature reported values (5-15 kcal mol^{-1}) for the lipase catalyzed transesterification reactions (Pogaku *et al.*, 2012).

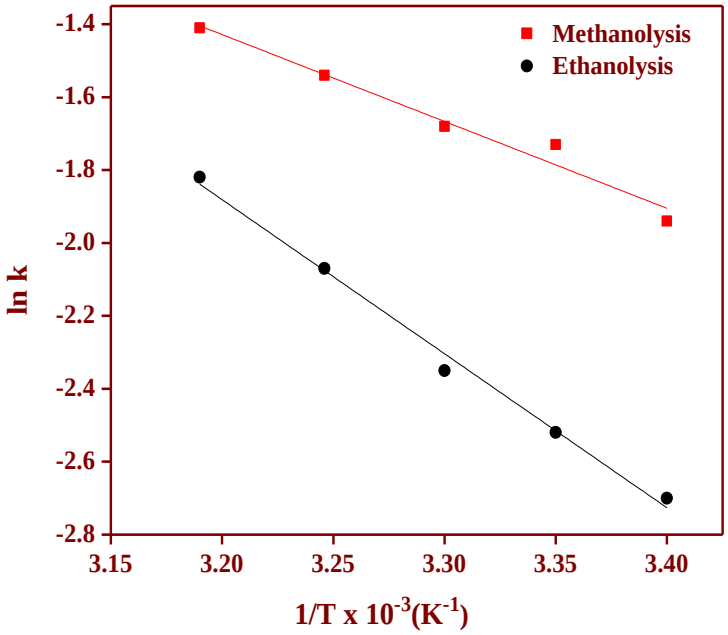

Figure 5.11. Arrhenius plot for the RML@SiO_2 catalyzed transesterification of CSO.

5.4 Conclusions

Rhizomucor miehei lipase was entrapped with in silica nanoparticles having oleic acid core (RML@SiO_2) in a single step and employed as solid biocatalyst for the transesterification of the cotton seed oil with methanol as well as ethanol. The enzyme activity was found to be a function of reaction temperature, and alcohol to oil molar ratio. However, immobilized enzyme didn't require buffer solution of any specific pH or organic solvent to yield the complete conversion of oil into alkyl esters. Under optimized reaction conditions RML@SiO_2 catalyzed ethanolysis and methanolysis has followed the pseudo first order rate law. The immobilized enzyme did not require the low temperature storage condition and the enzyme stored even at room temperature (25-30 °C) was found to be stable over a period of 8 months. The immobilized enzyme has also demonstrated excellent reusability as it was employed successfully in twelve transesterification reaction cycles without significant loss in activity.

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Chapter-6

Conclusions and futuristic aspect

Abstract: The results given in chapter 3, 4 and 5 have been concluded, compared and correlated in this chapter. Further studies possible with the immobilized enzymes have also been proposed at the end of chapter.

6.1 Introduction

In present thesis lipase enzyme has been used as biocatalysts for the transesterification reaction of vegetable oils. In chapter 3, activity of pure and immobilized lipase has been evaluated in presence of metal ions. While in chapter 4 and 5, lipase has been immobilized over solid support to obtain reusable solid biocatalyst. Reusability of the immobilized lipase may reduce the effective cost of lipase. Additionally, the solid biocatalysts are expected to produce the biodiesel and glycerol without any contamination.

6.2 Conclusions from the present work

i. In comparison to the chemical catalyzed transesterification reactions, lipase catalyzed reactions are slower and hence, required longer reaction duration to achieve acceptable FAMEs yield (> 96.5 %).

ii. **In chapter 3,** attempts are made to enhance the hydrolytic and transesterification activity of *Candida rugosa* lipase (CRL) by adding alkali (Na^{+} and K^{+}), alkaline earth (Ca^{+2} and Ba^{+2}) and transition (Cr^{+3}, Fe^{+3}, Co^{+2}, Cu^{+2} and Ni^{+2}) metal ions in the enzyme assay.

iii. Maximum enhancement in lipase hydrolytic activity was observed by Ca^{+2}, and in transesterification activity by Cr^{+3} and Co^{+2} and time required for the complete ethanolysis and methanolysis of cotton seed oil was reduced from ~ 52 and 48 h, respectively to 22 h.

iv. The kinetics of the lipase catalyzed transesterification (methanolysis and ethanolysis) reactions were also studied, and the activation energies of methanolysis and ethanolysis were reduced from 10.13 and 10.24 kcal mol^{-1} to 5.15 and 7.32 kcal mol^{-1}, respectively, when reactions were performed in presence of Co^{+2}.

v. The activity of the commercially available immobilized enzymes was not found to be positively influenced due to the presence of metal ions.

vi. As discussed in chapter 3, although the activity of the lipase was enhanced by adding metal ions in the reaction assay, but enzyme once used could not be recycled.

vii. **In chapter 4,** in order to prepare the reusable solid biocatalyst, MCM-41, SBA-15 and Ti/SiO_2 has been prepared and used as a support for the immobilization of *Candida rugosa* lipase (CRL) by the physical adsorption technique.

viii. The enzyme immobilization was found to be a function of pH of the adsorbing medium, contact time and type of support.

ix. The prepared solid biocatalysts were employed as solid biocatalyst for the transesterification of cotton seed oil with methanol, and best activity was demonstrated by CRL-MCM-41. A 98% FAMEs yield was obtained in presence of 5 wt% (with respect to oil) CRL-MCM-41, using a 12:1 methanol to oil molar ratio at 40 ºC and 8 pH.

x. Immobilized enzyme was recovered after the completion of the reaction, however only partial conversion of oil into FAMEs was achieved in second and third catalytic runs.

xi. **In chapter 5,** in order to further enhance the stability and reusability of the immobilized enzyme, *Rhizomucor miehei* lipase (RML) has been entrapped in silica nanoparticles ($RML@SiO_2$), having oleic acid core, in a single step.

xii. The characterization of $RML@SiO_2$ by FTIR and TEM technique supported the entrapment of the lipase within silica particle.

xiii. The immobilized enzyme was able to catalyze the ethanolysis as well as methanolysis of cotton seed oil and activation energy for these reactions were found to be 8.36 and 4.72 kcal mol^{-1}, respectively

xiv. The enzyme was amenable to recovery and reuse for the twelve successive cycles and found to be stable and active even after eight months of storage at room temperature.

6.3 Futuristic aspects

i. In present thesis, effect of only metal nitrates have been evaluated on hydrolysis and transesterification activity of *Candida rugosa* lipase. In order to establish a particular trend/correlation between metal-lipase interaction and activity, the

study need to be performed in presence of other metal salts (*e.g.*, sulphates, chlorides, phosphates, etc.) with other lipases as well.

ii. The activity of the lipase immobilized catalyzed reactions are still much lower than the chemical catalyzed reactions. Hence, it is necessary to find out the ways to enhance the rate of lipase catalyzed reactions.

iii. In the present work only two immobilization techniques, such as physical adsorption and entrapment, are employed using mesoporous silica as matrix. In near future other immobilized techniques could be employed for the lipase immobilization over polymeric support.

iv. Since lipase can act on a variety of substrates, it would be interesting to study the activity of the immobilized lipase towards the esterification, aminolysis and hydrolysis reaction using variety of substrates.

List of publications

1. Katiyar, M.; Ali, A. Immobilization of *Candida rugosa* lipase on MCM-41 for the Transesterification of cotton seed oil. *J. Oleo Sci*. **61**, 469-475 (2012).

2. Katiyar, M.; Ali, A. Effect of metal ions on the hydrolytic and transesterification activities of *Candida rugosa* lipase. *J. Oleo Sci*. **62**, 919-924 (2013).

Manuscript under preparation

1. Madhu katiyar and Amjad Ali *"*One pot entrapment of lipase in silica nanoparticles with oleic acid core: kinetics of transesterification, enzyme stability and reusability studies" manuscript submitted in J.Mol. Catal. B: Enzym.

2. Madhu katiyar and Amjad Ali *"*Transesterification of cotton seed oil using SBA-15 immobilized *Candida rugosa* lipase" manuscript under preparation submission.

Publications in Conferences

1. Amjad Ali, Prual Saraswat and Madhu Katiyar, Effect of metal ions and organic molecules on lipase catalyzed hydrolysis of *p*-nitrophenyllaurat, National symposium on Green Chemistry: Applications in Science & Engineering (NSGC-2009) February 5-6, 2009 organized by School of Chemistry & Biochemistry, Thapar University-Patiala.

2. Madhu Katiyar and Amjad Ali, Effect of metal ions on transesterification activity of commercially available immobilized lipase, National Conference on Innovative Molecules for Sustainable Future (NCIMSF-2013) 24-26 October, 2013 organized by School of Chemistry and Biochemistry, Thapar University-Patiala.