Enzymatic Esterification of Oleic Acid

A dissertation submitted

in partial fulfillment for the award for the

degree of

Master of Science
in
Chemistry

Submitted by

Meenakshi Rani

(Reg. No. 300802010)

Under Supervision of

Dr. Ranjana Prakash

School of Chemistry and Biochemistry
Thapar University
Patiala

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Candidate's Declaration

I hereby declare that the work which is being presented in the dissertation entitled **"Enzymatic esterification of oleic acid"** in partial fulfillment of the requirement for the award of the degree of **Master of Science in Chemistry**, School of Chemistry and Biochemistry, Thapar University, Patiala is an authentic record to my own work carried out during a period of six months from January 2010 to June 2010, under the supervision of Dr. Ranjana Prakash, School of Chemistry and Biochemistry, Thapar University, Patiala. I have not submitted the matter embodied in this dissertation for the award of any other degree or diploma.

Place: Patiala
Date 15/07/10

Meenakshi Rani

Meenakshi Rani

This is to certify that the above statement made by the Candidate is correct and true to best of our knowledge

Dr Ranjana Prakash
Assistant Professor
School of Chemistry & Biochemistry
Thapar University, Patiala

Susheel Mittal 15/7/10

Dr. Susheel Mittal
Head
School of Chemistry & Biochemistry
Thapar University, Patiala

Certificate

This is to certify that the project entitled **"Enzymatic esterification of oleic acid"** being submitted by Miss. **Meenakshi Rani** in partial fulfillment of the requirement for the award of degree for the Master of Science in Chemistry at the School of Chemistry, Thapar University, Patiala, is a bonafied work carried out under my guidance and supervision and that no part of this project has been submitted for the award of any other degree.

Dr Ranjana Prakash
Assistant Professor
School of Chemistry & Biochemistry
Thapar University, Patiala

Dr. Susheel Mittal 15/7/10
Head
School of Chemistry and Biochemistry
Thapar University, Patiala

Dr. R.K.Sharma 16.7.10
Dean
Thapar University
Patiala

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Meenakshi Rani

Summary

The study entitled "**Enzymatic esterification of oleic acid**" was focused to examine the potential of lipases sourced from *Candida rugosa* and *Aspergillus niger* towards esterification of oleic acid in the presence of various alcohols as reactants. The study was carried in both aqueous and non-aqueous conditions. The salient findings of the study include (a) near complete esterification of oleic acid to ethyl oleate by *C. rugosa* lipase (b) significant yield of alkyl esters in aqueous conditions; (c) significant yield of pentyl oleate in reaction catalyzed by lipase sourced from either of the organisms; (d) increasing trend in yield of alkyl esters with increase in chain length of alcohols upto pentanol in aqueous system with *C. rugosa* lipase.

Contents

Chapter 1

Introduction

Esterification is the chemical process for making esters, which are compounds of the chemical structure R-COOR', where R and R' are either alkyl or aryl groups. The most common method for preparing esters is to heat a carboxylic acid, R-CO-OH, with alcohol R'-OH, in the presence of catalyst. Different types of catalyst are used for the esterification reacion due to its industrial importance. Short chain esters are used in flavour and fragrance industry on the other hand long chain ester are used in wax and fuel industry. For eserification reaction to be catalyzed, chemical or biological catalyst are used. Chemical catalysis is carried out either by acid or base, at high temperatures (200–250ºC) and pressures (Kaufman and Garti 1982). This chemical reaction is tedious, nonselective and consumes large amount of energy. The product obtained has to be purified further, by alkali washing, steam refining, molecular distillation, ultra filtration, activated carbon treatment, etc. Some time side reaction like saponification occurs if moisture is present in the reaction mixture. On the other hand biocatalysts, like lipases can be used for esterification reaction. Furthermore easy biocatalyst recovery, recyclability and possibility of continuous operation as well as improved stability of the biocatalysts make its use at industrial level.

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) received much attention in the fields of organic chemistry, biotechnology, food and pharmaceutical chemistry owing to ubiquity, broad substrate specificities, requirement of mild reaction conditions and cause less environmental pollution (Zheng and Yan 2004). They have been widely used for enzymatic bio-transformation of oils/fats and ester synthesis (Deng *et al.,* 2003). Recently, synthesis of fatty acid alkyl esters received much attention since esters produced from long-chain fatty acids (12–20 carbon atoms) and short-chain alcohols (3–8 carbon atoms) have been used increasingly in the food, detergent, cosmetic, pharmaceutical and fuel industries. The modification of structure and composition of oils and fats by the enzymatic esterification has become greater interest in the food industries. The lipolytic reactions in the non-aquous

media has been indicated as novel approach for the production of the nutrients, pharmaceuticals and several other basic materials which find enormous application in the industries (Zaks and Klibanov 1988).

Lipases stand amongst the most important biocatalysts carrying out novel reactions in both aqueous and non-aqueous media. This is primarily due to their ability to utilize a wide spectrum of substrates, high stability towards extremes of temperature, pH, organic solvents, and chemo, region and enantioselectivity. Lipases can be further exploited in many newer areas where they can serve as potential biocatalysts. Ester synthesis by lipases can be performed at room temperature and pressure, providing an energy-saving procedure under neutral pH in reaction vessels operated either batchwise or continuously (Lai and O'Connor 1999). Therefore, the products obtained are qualitatively purer than those obtained by chemical catalysis, which tends to be unspecific and consequently generates several by products (Roy and Bhattacharya 1993). The use of lipases to carry out esterification alleviates the necessity of a wide variety of complex post-reaction separation processes and thus leads to lower overall operation costs (Yahya *et al.,* 1998).

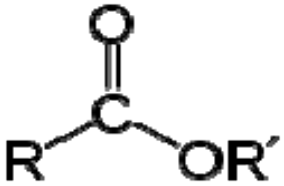
The esterification by lipases appears to be an attractive alternate to the bulk chemical routes. Lipases are being developed to carryout the transformations without extreme temperature and pressure which are essential for traditional chemical processes. The low temperature biocatalysed esterification processes has many advantages over conventional chemical methods. Besides the development of economical processes, the environment friendly nature of enzymatic production methods also offers obvious advantages. Reduction in energy usage and disposal of waste materials generated are points to note in estabilishing ecologically favourable "green" sustainable technology.

Keeping in view the present study was carried out to standardize and optimize biocatalyzed esterification reaction using lipase sourced from *Candida rugosa* in aqueous medium.

Chapter 2

Review of Literature

Esters are chemical compounds derived by reacting of an oxoacid (one containing an oxo group, X=O) with a hydroxyl compound such as an alcohol or phenol (McNaught and Wilkinson,1997) and is named as esterification reaction. Esters are usually derived from an inorganic acid or organic acid in which at least one -OH (hydroxyl) group is replaced by an -O-alkyl (alkoxy) group, and most commonly from carboxylic acids and alcohols.



Esterification

It is a general term used for a chemical reaction in which two reactants (typically an alcohol and an acid) form an ester as the reaction product. It may be catalyzed by acids, bases or enzymes. Generally, a mole of acid reacts with a mole of alcohol to give one mole of ester and at the same time one mole of water is released. Esterification can be represented by following reaction:

$$\underset{\text{Carboxylic acid}}{RCOOH} + \underset{\text{Alcohol}}{R'OH} \rightarrow \underset{\text{Ester}}{RCOOR'} + H_2O$$

Esters can also be formed by various other reactions. These include the reaction of an alcohol with an acid chloride (R-CO-Cl) or an anhydride (R-CO-O-COR'). Earlier studies on the chemical mechanism of esterification, concluded that the ester product (R-CO-OR') is the union of the acyl group (R-C=O) from the acid, *RCO*-OH, with the alkoxide group (R'O-) from the alcohol, *R'-O*H rather than other possible combinations. The chemical structure of

the alcohol, the acid, and the acid catalyst used in the esterification reaction all effect the rate of esterification reaction. Simple alcohols such as methanol (CH_3OH) and ethanol (CH_3CH_2OH) react very fast because they are relatively small and contain no carbon atom sidechains that would hinder their reaction. Simple acids such as acetic acid or vinegar (CH_3CO_2H) also form esters very easily. Esterification is highly reversible, however, the yield of the product may be improved using le Chatelier's principle

- using the alcohol in excess (i.e. as a solvent),
- using a dehydrating agent. (Sulfuric acid not only catalyzes the reaction but sequesters water, a reaction by-product. Other drying agents like molecular sieves can also be used.)
- removal of water by physical means such as distillation as a low-boiling azeotropes with toluene.

The esterification process has a broad spectrum of uses from the preparation of highly specialized esters in the chemical laboratory to the production of millions of tons of commercial ester products. Among different esters, fatty acid alkyl esters (known as biodiesel), which is derived from triglycerides or free fatty acids by esterification or transeterification with short chain alcohols, has attracted considerable attention during the past decades as a renewable, biodegradable, and nontoxic fuel. Fatty acid esters of sugars and sugar alcohols find widespread applications as surfactants/emulsifiers in food, detergents, cosmetics and pharmaceutical industries owing to their biodegradability and low toxicity (Piccicuto *et al.*, 2001, Saxena *et al.*, 1999). Similarly, alcoholic esters of short chain fatty acids are important flavor and aroma compounds, whereas esters of long chain fatty acids are being explored for their use as fuel biodiesel and as waxes (Saxena *et al.*, 1999) in the oleo-chemical industries. Among these, the fatty acid esters of sorbitol are the second largest class of carboxylic acid esters employed as surfactants (Arcos *et al.,* 1998).

Catalysts for esterification reaction

Chemical catalysts

Acids can be esterified by alcohols in the presence of a suitable acidic catalyst. The initial step is protonation of the acid to give a cation (Abel *et al.,* 1963), which can undergo an exchange reaction with an alcohol to give the intermediate, and this in turn can lose a proton to become an ester (Ackman *et al.*, 1969). Each step in the process is reversible but in the presence of a higher excess of the alcohol, the equilibrium point of the reaction is displaced so that esterification proceeds virtually to completion. However, in the presence of water, which is a stronger electron donor than are aliphatic alcohols, formation of the intermediate (Ackman *et al.*, 1977) is not favoured and esterification is not complete. Esterification, using mineral acids, is of limited use due to the reversibility of reactions, length of reaction time and above all the undesirable side reactions with other units of the reacting fatty acids. Preparation of higher esters by purely chemical means requires higher energy consumption of acid catalysts and result in the formation of some toxic and coloured by products (Saxena *et al.*, 1999). Esterification in the presence of base such as sodium hydroxide, potassium hydroxide, carbonates and alkoxides such as sodium methoxide and sodium butoxide, proceeds at much faster rate than catalysed by same amount of acid catalyst.

These catalysts have inherent drawbacks such as the requirement for excess reagents, corrosiveness and high susceptibility to water, difficulty in catalyst recovery and reuse, being more energy intensive and posing environmental hazards. The most effective tool to deal with the problem is the use of less energy intensive and eco-friendly enzyme catalyst for esterification.

Enzyme Catalysis

At present, there is considerable interest in the use of enzymes as catalysts in organic synthesis. (Wong 1989, Waldmann *et al.*, 1994, Hayes 1996, Anderson *et al.*, 1998, Bornscheuer 2000, Faber *et al.*, 2000, Liese *et al.,* 2000, Miller 2000). Among the reactions that are studied are hydrolysis, esterification, and transesterification catalyzed by hydrolytic enzymes such as lipases, esterases, and proteinases. Esterification by lipases appears to be an

attractive alternative to bulk chemical routes. Among biocatalysts, lipase is used for esterification to a significant extent. Lipases are a class of enzymes known as triacylglycerol ester hydrolases. Lipase may be used in the reaction in different ways:

- Pure lipase along with acid and alcohol in the reaction mixture.
- Lipases immobilized on diversed variety of matrices.
- Lipase producing free whole cell, and
- Immobilized microbial strains having potential to produce lipase in specific culture conditions.

Lipases are the hydrolase group of enzymes, which catalyze the hydrolysis of glyceride ester bonds. The fatty acid released is transformed to water or molecules having a free hydroxyl group or related moiety (nucleophile). They are also termed as acylglycerolases, acyl hydrolases, or triacylglycerol hydrolases. Lipases, when employed to catalyze esterification and transesterification reactions in organic solvents, have shown pH memory (Zaks and Klibanov 1985, Zaks and Klibanov 1988) increased enzyme activity and stability at elevated temperatures (Zaks and Klibanov 1984, Ahern and Kalibanov 1985) regiospecificity and stereoselectivity and may be affected by water activity (Goderis *et al.*, 1987). Most importantly, lipases do no require cofactors for activity.

Many microorganisms (bacteria, yeast and fungi) have been observed to produce lipases during growth in hydrophobic substrates (Haba *et al.*, 2000). Lipases found from *Mucor miehei, Rhizopus oryzae, Candida antartica* and *Pseudomonas cepacia* were found to be suitable for biodiesel (Iso *et al.*, 2001, Du *et al.*, 2008). Among lipases of various sources such as animal, plant, microbes and those from yeast, enzymes particularly from *Candida sp.* show highest degree of versatility, reactivity and stability in the catalysis of reactions in organic phase. Among the yeasts, *C.rugosa* was identified as potent producer of lipase (Akoh *et al.* 2004). The broad synthetic potential of lipases is largely due to the fact that they (in contrast to most other enzymes) accept a wide range of substrates and tolerate organic solvents. They can be employed for either hydrolysis or synthetic reactions depending on the solvent system. A wide range of substrates other than triglycerides (such as aliphatic, alicyclic, bicyclic and aromatic esters, and even esters based on organometallic sandwich

compounds) are accepted by lipases. When the racemic esters or substrates with several hydroxyl groups are fed, lipases react with a high degree of enantio- and regioselectivity. A wide range of thioesters and activated amines can also be substrates for lipases, which expands their potential tremendously (Faber 2000).

Ester synthesis reactions make use of the law of mass action to drive the equilibrium in the direction of the synthesis by removing the water generated during the reaction. Ester exchange reactions take place at low water activity (Macrae 1983). The optimum yield in the esterification was obtained by determining the optimal substrate and enzyme concentration. The inhibiting effects of the acids were strongly attenuated by reducing the quantity of the acid and increasing the amount of enzyme in the media (Laboret and Perraud 1999). The esterification performance was dependent on the alcohol structure, with maximum activity occurring for the primary alcohol. Secondary and tertiary alcohols decreased the reaction rates by more than 40%. The ester synthesis can be maximized for the substrates containing the excess acyl donors (Bruno *et al.,* 2004). (Garcia *et al.,*2002) developed a methodology for the *esterification* of an acid with an epoxide using 2- chlorobutyric acid and 1,2-epoxy-5 hexene catalyzed by a *Mucor miehei* - immobilized lipase. This could be applied to obtain 2-chloroesters. The esterification of cinnamic acid and oleyl alcohol in the organic solvent media by the immobilized lipase Novazym 435 was optimized with a bioconversion of 100% after 12 days of reaction. The electrospray ionization mass spectroscopy analysis confirmed that the major end-product of the esterification reaction was oleyl cinnamate.

Lipase mediated ester synthesis can be performed at the room temperature, pressure and with neutral pH in reaction vessels operated either batchwise or continuously. The effect of fatty acid chain length on *C. antarctica* lipase B (Novazym 435) catalyzed sugar esterification was studied in a mixture of 9:11, *t*-Butanol and pyridine (v/v). a and b maltose 6'O-acyl esters in an anomeric molar ratio of 1.0:1.1 were synthesized independently of the chain length, but the initial specific reaction rate increased with the chain length, of the acyl donar. The highest initial reaction rate and yield were obtained with the shortest chain length of the acyl donar (Pedersen *et al.*, 2002). The immobilized lipase from *C. antarctica* was applied to perform the enzymatic esterification of the bioactive compounds with the fatty acids pyridoxine condensate with lauric acid was studied as the model system and lauric acid

pyridoxine mono esters with high retention time was obtained as the product. For the esterification between the hydroxyl group of lactic acid and the carboxyl group of organic acids, Kiran and Divakar (2001) employed lipozyme IM20 from *Rhizomucor miehei* and porcine pancreas. The reactions were carried out at both shake-flask and bench-scale levels. At the shake-flask level, maximum yields of 37.5 and 40% were observed in case of palmitoyl and stearoyl lactic acids, respectively, with Lipozyme IM20; at bench-scale level, the maximum yields were 85.1 and 99% respectively, when porcine pancreas lipase was employed. Moreover, this could be reused for three cycles with yield above 40%. The esters prepared were found to confirm the food chemical codex (FCC) specifications in terms of the acid value, ester value, sodium and lactic acid contents.

Lipase catalyzed formation of isoamyl bulyrate was investigated as model to study the kinetics of lipase–catalyzed esterification. The reaction rate was described in terms of the Michaelis-Menten equation with a Ping-Pong Bi-Bi Mechanism (Krishna *et al.,* 2001). In this reaction, the increase in butyric acid concentration in microaqueous layer caused a pH drop in the enzyme microenvironment, leading to enzyme inactivation. Enantioselective resolution of trans-2-phenyl-1- cyclohexanol (TPCH) by a *C. rugosa* lipase was obtained by the esterification of the immobilized EP100 polypropylene powder using isooctane as the solvent and propionic acid as the esterifying agent. High value of conversion and enantioselectivity was observed in the continuous packed bed reactor. The TPCH, thus, produced has been used in the synthesis of wide variety of chiral molecules such as hydroxy aminoacids, diltiazen etc. due to its versality and a high level of stereo control (Sanchez *et al.*, 2000). Legrand *et al.* (1990) checked thirteen commercial lipase preparations for their ability to catalyze the formation of flavour esters. Acetic acid esters were more difficult to obtain than isomyl, gernoyl acetate, propionate and butyrate.

The advantages mentioned earlier enable their use in the synthesis of certain specialty chemicals and pharmaceutical intermediates. The use of lipases in esterification reactions is also extended to generation of industrially important products such as emulsifiers, surfactants, wax esters, chiral molecules, biopolymers, modified fats and oils, structured lipids, flavor esters and esters of long chain fatty acids.

Esters of long chain fatty acid are commonly used as biodiesel, number of enzymes are employed for esterification of long chain fatty acid and short chain alcohols (Fukuda *et al.,* 2001). Biodiesel used as replacement of fossil fuels to secure future energy supplies continues to be a major concern. In this connection, biodiesel (Knothe *et al.*, 2005) is an alternative to petroleum-based diesel fuel (petrodiesel). Biodiesel is the methyl (or ethyl) ester of the fatty acids found in vegetable oils or animal fats. At the simplest, these fats are *triglycerides*. The most common fatty esters contained in biodiesel are palmitic (hexadecanoic) acid, stearic (octadecanoic) acid, oleic (octadecenoic) acid, linoleic (octadecadienoic) acid, and linolenic (octadecatrienoic) acid.

The global biodiesel industry has grown significantly over the past decade. Some of the main drivers behind the tremendous growth are reducing dependence on imported oil, environment friendly alternative to diesel, reducing greenhouse gas emissions, can be used in the existing diesel engines with limited modifications. The attractive features of biodiesel are (Fukuda *et al.*, 2001):

- It is plant derived and its combustion does not increase current net atmospheric levels of CO_2. In addition, relative to conventional diesel fuel, its combustion products have reduced levels of particulates, carbon monoxide, $S0_2$ and under some conditions, nitrogen oxides
- It is biodegradable.

Keeping in view, the potential of esterification reaction in production of customized esters, the present study was focused on process optimization for esterification of oleic acid with short chain alcohol methanol, ethanol, propanol,butanol, pentanol, hexanol, heptanol, octanol, nonanol, decanol) by using pure lipase of *Candida rugosa.*and *Aspergillus niger.*

Chapter 3
Materials and Methods

Materials

Pure lipases of *Candida rugosa* and *Aspergillus niger* were sourced from Sigma-Aldrich. Other chemicals such as oleic acid, different short chain alcohols (methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol and decanol), hexane, ethyl acetate and silica gel G, used during the study were procured from SD Fine Chem. Pvt.Ltd.

Methods

Following parameters were modulated for esterification of oleic acid

1. enzyme concentration of *Candida rugosa* in aqueous and non-aqueous medium; and
2. alcohols having varying chain length as reactants.

The observations on influence of chain length of alcohols were also studied with *A.niger* lipase with reference to yield of esterification products, based on the optimal parameters determined with studies on *C.rugosa* lipase.

A. Optimization of enzyme concentration of *Candida rugosa* in aqueous and non-aqueous medium for better reaction yield

i. In aqueous medium

The potential of pure lipases of *Candida rugosa* to exhibit catalysis was determined by dissolving of 1gm of lipase in 10ml of distilled water followed by preparing different ratios (1:9, 2:8, 3:7, 4:6, 5:5 and 6:4) of enzyme to oleic acid using the above solution. Reaction mixture was taken in 50 ml round bottom flask immersed in water bath on magnetic stirrer set at 35º C and maintained for 24 h. Product obtained was analyzed using thin layer chromatography (TLC) followed by confirmation using ^{1}H nuclear magnetic resonance (NMR) spectrometer.

ii. In non-aqueous medium

Series of experiments were carried out to optimize the esterification of oleic acid by using pure lipase in non-aqueous systems. Pure lipase of *C.rugosa* was directly suspended in oleic acid at varying concentrations ranging from 0.5g (5%) to 4.0g (40%) in 10ml oleic acid so as to optimize the yield of ethyl ester. The reaction was carried out in 20 ml test tube immersed in water bath set at 35º C and maintained for 24h. Product obtained was analyzed using thin layer chromatography (TLC) followed by confirmation using ^{1}H nuclear magnetic resonance (NMR) spectrometer.

Role of alcohol chain length on yield of esterification

Based on the optimal (a) yield of alkyl esters in aqueous medium and (b) oleic acid to enzyme concentration ratio, further study was carried out to understand the influence of chain length of different alcohols (methanol, ethanol, propanol, butanol, pentanol and hexanol). 1:1 molar ratio of enzyme to acid was taken in 50ml round bottom flask and kept in water bath set at 35º C for duration of 1h to facilitate mixing of reactants. Alcohol was added to the reaction mixture, in 1:1 molar ratio (acid:alcohol), through stepwise addition after 1h, 4h and 7h of reaction. Reaction was carried out for 24 h. Product obtained was analyzed using thin layer chromatography (TLC) followed by confirmation using ^{1}H nuclear magnetic resonance (NMR) spectrometer.

B. Optimization of enzyme concentration of *Aspergillus niger* in aqueous medium for best possible yield of alkyl esters

The reaction conditions in aqueous medium, that facilitated optimal yield of alkyl esters in the presence of *C.rugosa* lipase as a catalyst, were used as a basis for this phase of the study. The study involved examining the effect of chain length of alcohols (ethanol to decanol) mentioned above on the yield of esterification using lipase sourced from *Aspergillus niger*.

Quantification of alkyl esters using ^{1}H NMR

Free fatty acid content was quantified by using formula given by Satyarthi *et al.* (2009). Quantification of the FFA content by ^{1}H NMR is based on the fact that α-CH_2 peaks

of fatty acids appear at δ values higher than those of the methyl (biodiesel) or glyceryl (vegetable oil) esters. The difference in chemical shift (between the acid and ester) is due to the greater deshielding effect of the carboxylic group compared to the ester group. Due to this shift, one of the peaks of the triplet of FFA (at 2.38 ppm) shifts out of the α-CH_2 region of the ester, and the other two peaks (2.34 and 2.30 ppm, respectively) are merged with those due to the ester at 2.35 and 2.31 ppm, respectively. In other words, a sample containing FFA and ester (vegetable oil or biodiesel) shows a quartet like spectral pattern in the α-CH_2 region of the ^{1}H NMR spectrum with the intensity of the peaks depending on the content of FFA in esters. The unmerged peak of the FFA triplet can be used to determine the FFA content (Figure 2b). The area (*A*FFA) of the unmerged peak of the FFA triplet (appearing around 2.38 ppm, out of the ester triplet) can be determined by integration of the spectral region 2.37-2.41 ppm. The triplet appears with an intensity ratio of 1:2:1. The total area corresponding to the α-CH_2 groups of the FFA, will thus, be four times the area of the single unmerged FFA peak around 2.38 ppm. The total area corresponding to α-CH_2 of both FFA and ester can be determined by integrating the spectral region 2.20-2.41 ppm. The concentration of FFA (wt %) in oil or biodiesel is thus

$$\%\ \text{of FFA} = \frac{4 \times \text{area of unmerged peak of } \alpha\text{-}CH_2 \text{ of FFA}}{\text{total area of } \alpha\text{-}CH_2 \text{ of both FFA and ester}} \times 100$$

Alternatively, the peaks due to the ester and acid can be de-convoluted, and the FFA content can be calculated using the given equation (Satyarthi et al. 2009).

$$\%\ \text{of FFA} = \frac{\text{area of triplet of } \alpha\text{-}CH_2 \text{ of FFA}}{\text{total area of } \alpha\text{-}CH_2 \text{ of both FFA and ester}} \times 100$$

The ^{1}H NMR spectral analysis was carried out at sophisticated analytical instrumentation facility (SAIF), Panjab University, Chandigarh.

Chapter 4

Results and Discussion

Lipases sourced from *Candida rugosa* and *Aspergillus niger* were examined for their potential to esterify oleic acid. The products, different alkyl esters from different alcohols, were analyzed by using thin layer chromatography (TLC) and further quantified by using H^1 NMR.

Optimization of enzyme concentration of *Candida rugosa* in aqueous and non-aqueous medium for esterification

Non-aqueous reaction

Initially, the concentration of lipase was optimized with respect to oleic acid and ethanol to carry out esterification reaction at 35ºC. Pure lipase was directly used to carry out esterification by varying its concentration in terms of percentage (with respect to oleic acid). The results indicated that with increase in concentration of enzyme, yield of the product increased from 44.25% (5%) to 97.66% (30%) which with further increase in concentration resulting in decreasing yield of the product. 89.15% (40%) (Fig. 1).

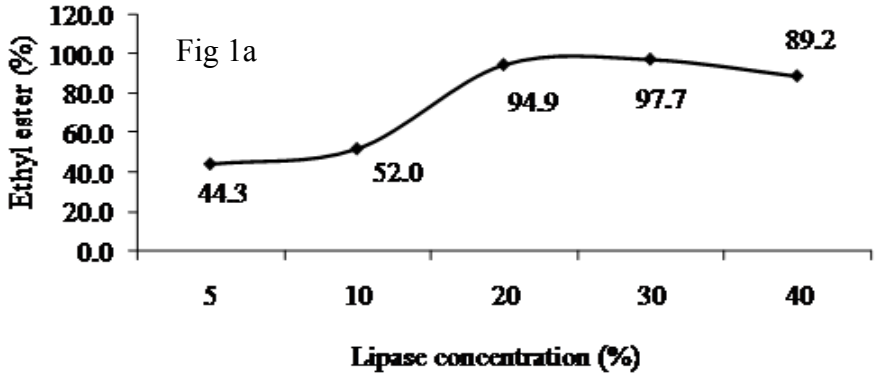


Fig 1b

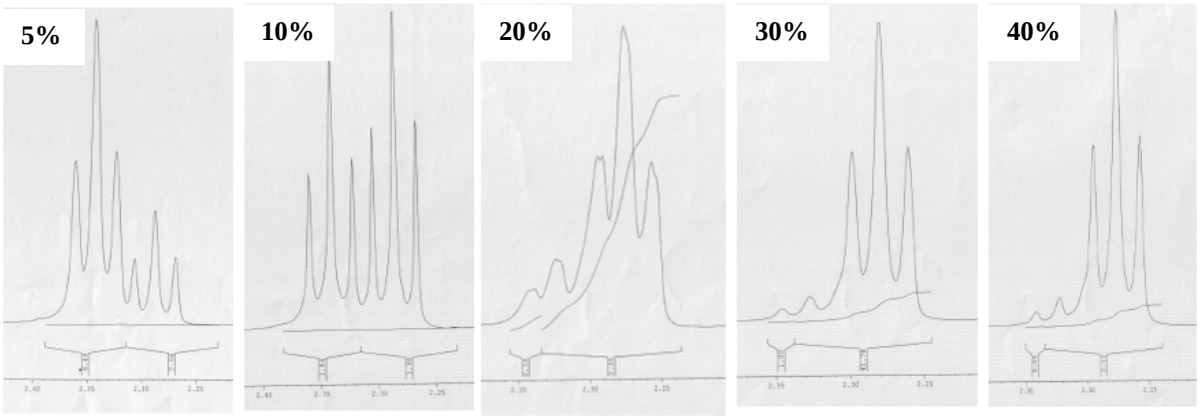


Figure 1 a&b. Effect of lipase concentration on the extent of esterification reaction. ^{1}H NMR of variations in the yield with increasing concentration of enzyme in non-aqueous condition

Radzi *et al.* (2005) studied the varying amount of enzyme on the esterification reaction of oleic acid and oleyl alcohol. The percentage conversion was observed to increase from 0.1 g (93.5%) to 0.4 g (95.4%) and remained constant with further increase in enzyme used beyond 0.5 g (95.1%). Garcia *et al.* (1999) investigated the influence of enzyme concentration during the esterification of palmatic acid with isopropyl alcohol using Novozyme 435. The studied values of enzyme concentration were 3, 5 and 7% (w/w). The results showed that the conversion increased with increasing enzyme concentration.

This phenomenon is explained by hypothesizing that, at high concentrations of enzyme the interfacial area is totally saturated with substrate. The observations on the excess biocatalyst not contributing to the increase in the percentage conversion is also further supported by Torres and Otero (2001) who reported that an excess of enzyme does not increase in percentage conversion and sometimes lead to decrease in the yield of the product.

Aqueous reaction

The optimization of enzyme concentration for esterification was carried out by using different ratios (1:9, 2:8, 3:7, 4:6, 5:5 and 6:4) of 10% enzyme solution with respect to oleic acid and ethanol. Results in table (2) indicated that with increase in enzyme dilution the extent of esterification increased from 23.5% (1:9) to 73% (6:4) (Fig. 2 a&b). To the best of our knowledge, such observation has not been reported hitherto in aqueous conditions.

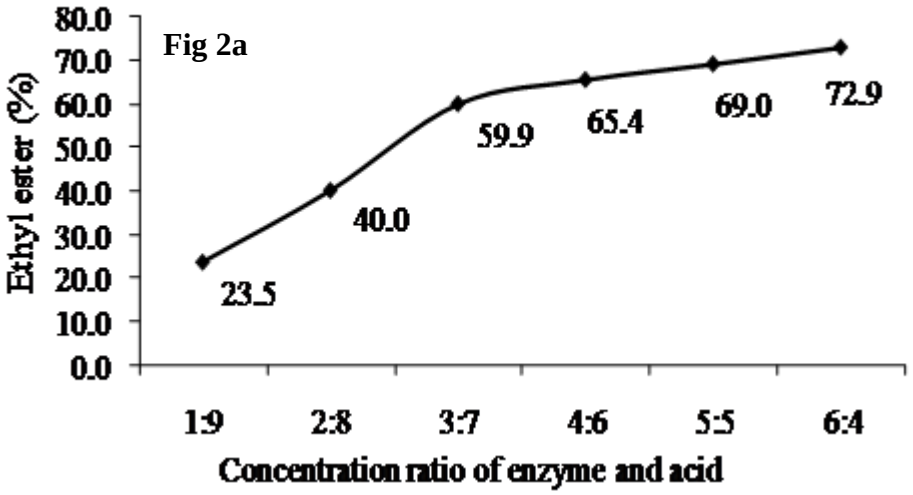


Fig 2b

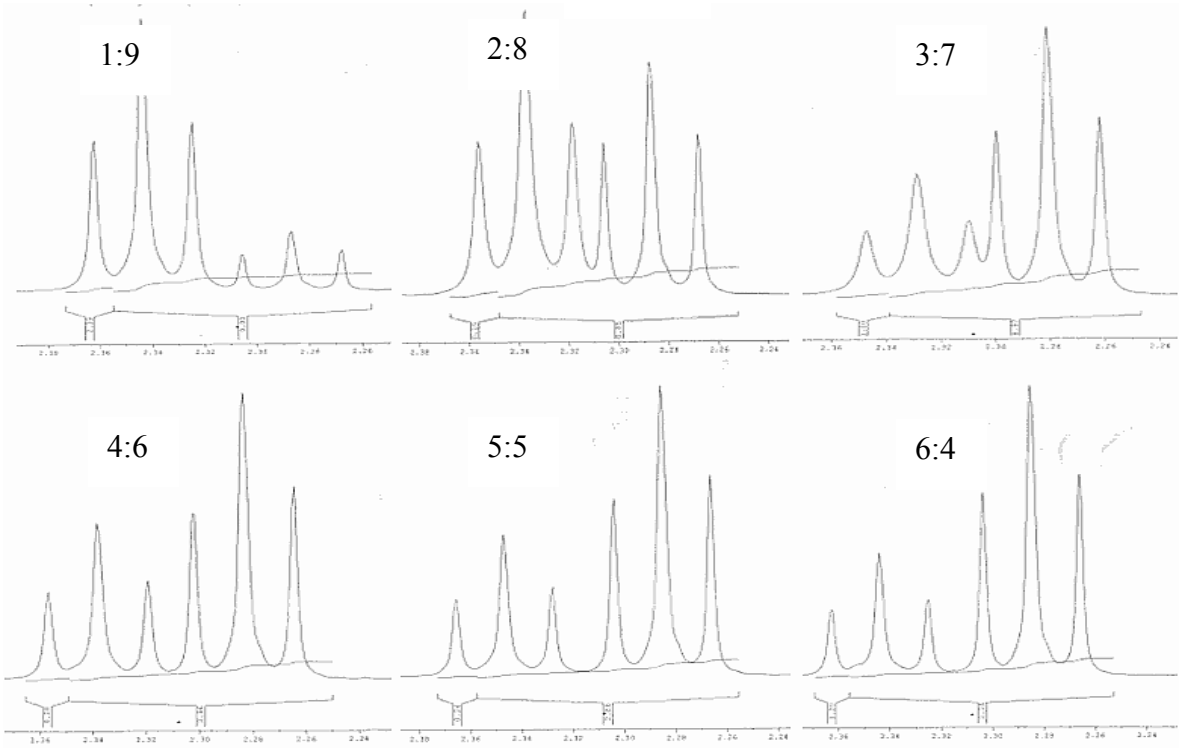


Fig 2 a&b. Effect of enzyme concentration on ethyl ester yield in aqueous condition. ^{1}H NMR of change in the yield with increasing concentration of enzyme over fatty acid

Influence of chain length of alcohol on esterification

Most of the previous reports in biocatalyzed esterification focused on variation in enzyme as a catalyst with limited observations on type of alcohol with reference to chain length. The optimal oleic acid to enzyme concentration ratio obtained in the present study was further used to catalyze the esterification of oleic acid with different alcohols with varying chain lengths viz., methanol, ethanol, propanol, butanol, pentanol and hexanol (Fig. 3a). The products thus obtained were analyzed using ^{1}H NMR (Fig. 3b).

Results in Fig 3a. indicate that with increase in chain length the extent of esterification increased from 64% (methanol) to 86% (pentanol) beyond which it decreased to 59.4% (hexanol).

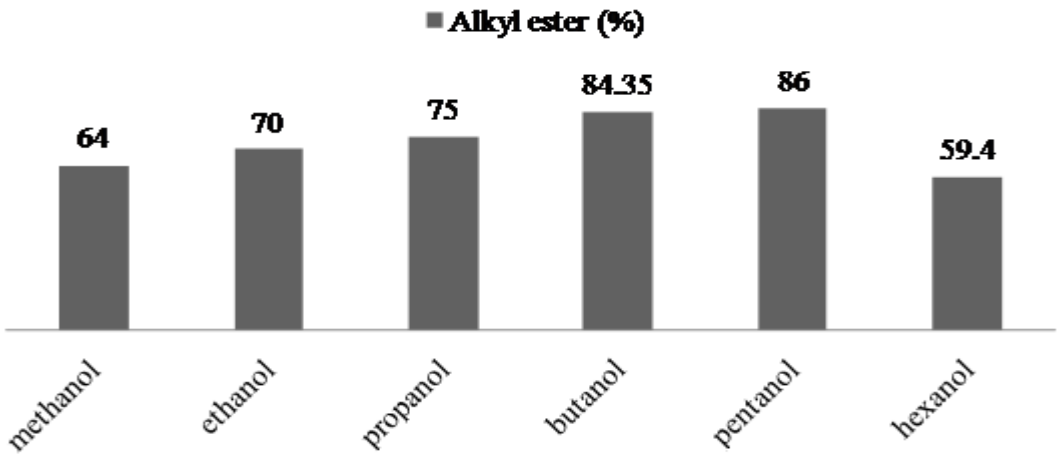


Fig 3a. Variations in the yield of alkyl esters (%) with various alcohols as reactants

Shimada *et al.* (1999, 2000) reported that methanol is observed to denature the enzyme thus decreasing its activity vis-à-vis the yield of the product. Esterification of oleic acid with different alcohols carried out using immobilized lipase from *Rhizomucor miehei* (Lipozyme IM) showed higher yield for the esterification with propanol when compared to other alcohols (Habulin *et al.* 1996). The authors reported that with further increase of the alcohol molar mass final conversion to alkyl esters observably decreased, thus supporting our observtions. Methanol is most commonly used to produce fatty acid methyl esters (FAME) (Akoh *et al.* 2007).

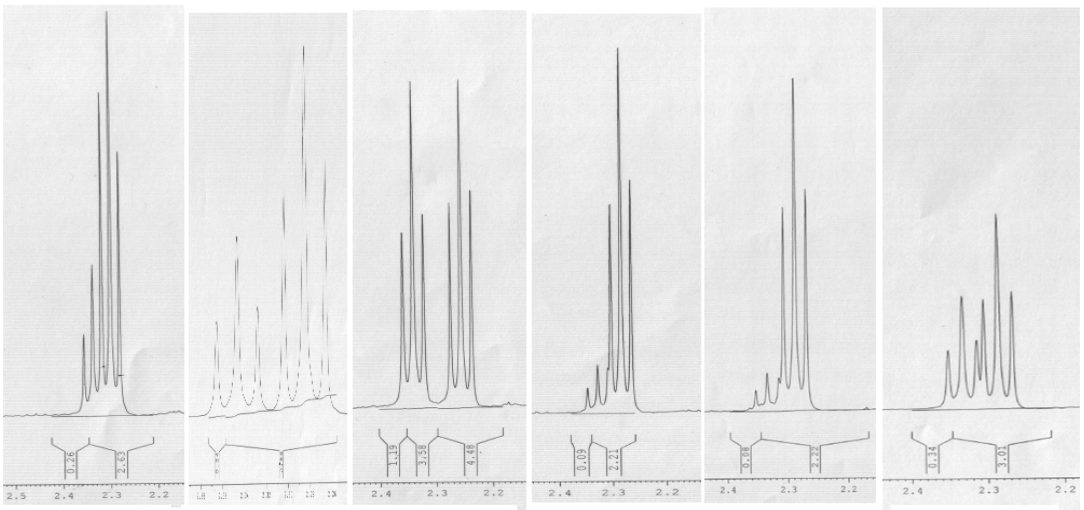


Figure 3b. ^{1}H NMR spectra of different alkyl esters (%)

Esterification of oleic acid with different chain length of alcohol by using lipase of *Aspergillus niger*.

Esterification reaction of oleic acid with different alcohols viz., ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol and decanol was carried out by using pure lipase of *Aspergillus niger*. The varying percentages of the yield of alkyl esters in the presence of above mentioned alcohols is presented in Fig. 4.

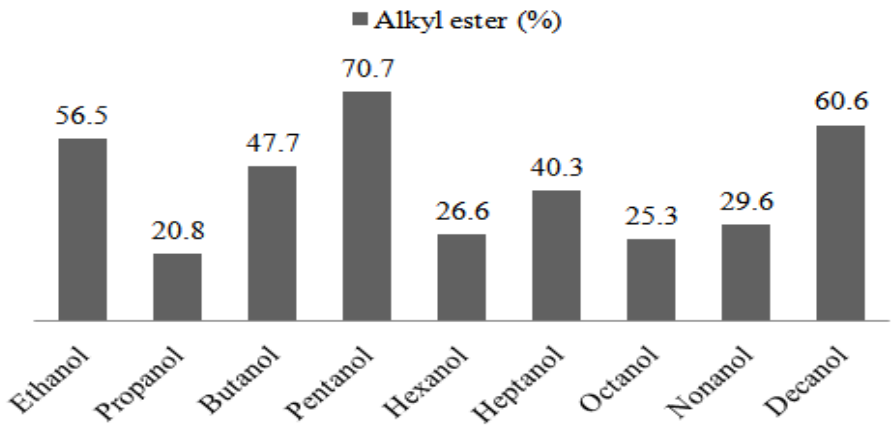


Fig 4. Variations in the yield of alkyl esters (%) with various alcohols as reactants

In comparison to the activity of lipase enzyme to different alcohols in case of *C.rugosa* wherein a definite trend could be observed with reference to the chain length of the alcohol, no such trend was evident in the case of lipase sourced from *A. niger.* A notable observation in the present study, is significant yield of pentyl oleate noted in catalytic reaction by both the lipase enzymes.

Esterification of oleic acid with different alcohols carried out using immobilized lipase from *Rhizomucor miehei* (Lipozyme IM) showed higher yield for the esterification with propanol when compared to other alcohols (Habulin *et al.,* 1996). The authors reported that with further increase of the alcohol molar mass final conversion to alkyl esters observably decreased, thus supporting our observations. Methanol is most commonly used to produce fatty acid methyl esters (FAME) (Akoh *et al.,* 2007). (Shimada *et al.*, 1999) reported that methanol is observed to denature the enzyme thus decreasing its activity vis-à-vis the yield of the product (Shimada *et al.*, 1999).

Ethanol is considered to be eco-friendly and is more renewable than methanol. Other acyl group acceptors that can be used for the alcoholysis reaction are ethanol, propanol, isopropanol (Shaw *et al.,* 1991, Lee *et al.,* 2004, Hernandez-Martin and Otero 2007), butanol, branched-chain alcohols (Nelson *et al.,* 1996, Modi *et al.,* 2006, Kose *et al.,* 2002), *t*-butanol (Royon *et al.,* 2007), and octanol (Marchetti *et al.*, 2007).

Conclusions

The present study, in general, demonstrated esterification of oleic acid in solvent and solvent-free system using different lipases sourced from *C.rugosa* and *A.niger*. In the case of non-aqueous system, a near complete esterification (98%), could be achieved in 24h with 30% enzyme concentration. Whereas, the reaction in aqueous system could result in maximum yield of 73% of ethyl ester. Esterification with different alcohols in optimized conditions resulted in maximum yield of pentyl oleate in case of lipase from either of organisms, where there was no similarity in the yield of other esters with these enzymes. The enzymatic esterification demonstrated in this study opens avenues to a greener synthesis of esters using microbial lipases.

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