STUDIES ON IMMOBILIZED LIPASE AND BIOSURFACTANT FOR HYDROLYSIS AND TRANSESTERIFICATION OF SUNFLOWER OIL

A thesis

Submitted in partial fulfillment of the requirement for the degree of

Doctor of Philosophy

by

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STATEMENT

I do hereby declare that the matter embodied in this dissertation is the result of investigations carried out by me in Centre for Energy, Indian Institute of Technology Guwahati under the guidance of Prof Pranab Goswami and Prof Pinakeswar Mahanta.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made, wherever the work described, based on the findings of other investigators.

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Abstract

The principal objective of this work is immobilization of lipase and application of the immobilized lipase for hydrolysis and transesterification of sunflower oil. Lipase was isolated from the culture supernatant of *Pseudomonas aeruginosa* PG01. A high activity (4.1±0.05U/ml) in the supernatant was detected when sunflower oil (2g/l) was used as the sole substrate. A significant production of lipase in burnt oil was observed, which was about 75% of the activity obtained when sunflower oil was used as the substrate. The extracellular lipase was successfully fractionated from the culture supernatant by ammonium-sulfate fractionation method, which was further purified by DEAE-sepharose anion exchange chromatography. The specific activity of the purified lipase was increased to 2.59 fold. The molecular weight of the DEAE sepharose filtered purified *Pseudomonas* lipase was estimated by SDS-PAGE was found to be 55 kDa. The optimum temperature and pH of the isolated lipase was 30°C and 7, respectively. The $t_{½}$ of the lipase at 4°C and pH 7 was nearly 23 months. The stability of the powdered lipase diminished steadily with the increasing temperature from 20 °C to 80 °C with $t_{½}$ of 62 days and 87 min at 20°C and 80°C, respectively. We report silk mat, a known biodegradable and renewable biomaterial, as support for the chemical immobilization of lipase. The chemically immobilized lipase was successfully used for the hydrolysis of sunflower oil for the production of fatty acids. This lipase catalyzed hydrolysis was found to occur effectively when the substrate sunflower oil was used as emulsion, mediated by either a chemical surface active agent, Triton X100, or a biological surface active agent, such as rhamnolipid. Further, we demonstrated that the rhamnolipid was a better surface-active agent than the Triton X100 for the hydrolysis, as evident from the 25.9% higher lipase activity realized by using emulsion created by biosurfactant over Triton X100. Although, the efficiency of the immobilized lipase was nearly 12% less than the free lipase on the biosurfactant-mediated

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hydrolysis of sunflower oil, the immobilized lipase could be reused even up to the third reaction cycle in this biosurfactant-mediated conversion. Hence this appears as potential industrial biocatalytic process for hydrolysis of vegetable oil. The production of methyl esters of fatty acids (biodiesel) from sunflower oil using immobilized lipase catalyzed transesterification reaction in a fabricated packed bed bio-reactor was investigated. Silk-cocoon was identified as novel and efficient support matrix for the lipase immobilization to carry out the reaction. The lipase was chemically immobilized on degummed and desericinated silk-cocoon by glutaraldehyde cross linking to a lipase loading density of 738.74U (or 0.22 g) /g silk-cocoon. The K_m of the immobilized lipase was 257.26 μM, which is nearly 47 % less than the K_m (451.26 μM) of the free lipase. Low K_m value of the immobilized lipase is attributed to the hydrophobic nature of the matrix that facilitated the substrates diffusion to the enzyme surface. A packed bed bio-reactor was successfully fabricated with the immobilized lipase for transesterification of sunflower oil for the production of biodiesel. The biodiesel yield of 81.62 % was obtained at 48 hours reaction time, 6:1 methanol: oil ratio (v/v) and 30°C. Whereas maximum biodiesel yield of 89% was obtained at 37°C under similar conditions. The conversion yield followed a third order polynomial (R^2= 0.95). The immobilized lipase showed high operational stability at 30°C. The substrate conversion was only marginally decreased till third cycle (each of 48 hours duration) of the reaction since less than even 5 % of the original activity was decreased in each of the second and third cycle. The findings demonstrated the potential of the silk-cocoon as lipase immobilization matrix for industrial production of biodiesel.

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CHAPTER

Introduction

1.1 Motivation

The rapid depletion of reserve to production ratio of mineral oil caused steep hikes in petroleum prices which prompted the researchers worldwide to explore biodiesel as a renewable transportation fuel and blending agent. Although the importance of biodiesel as a renewable and low emission fuel source has been overwhelmingly advocated in science and business world, the commercialization is largely stalled by the high cost of its production. In order to overcome the cost barrier, further investigation is warranted to develop an economically viable as well as environment friendly technology for biodiesel production process. The energy crisis of the world is thus met-up with this fruitful supplement of economically viable biodiesel process technology. However, the novelty of this technology can only be commercialized if we ensure its production and performances by economically feasible ways.

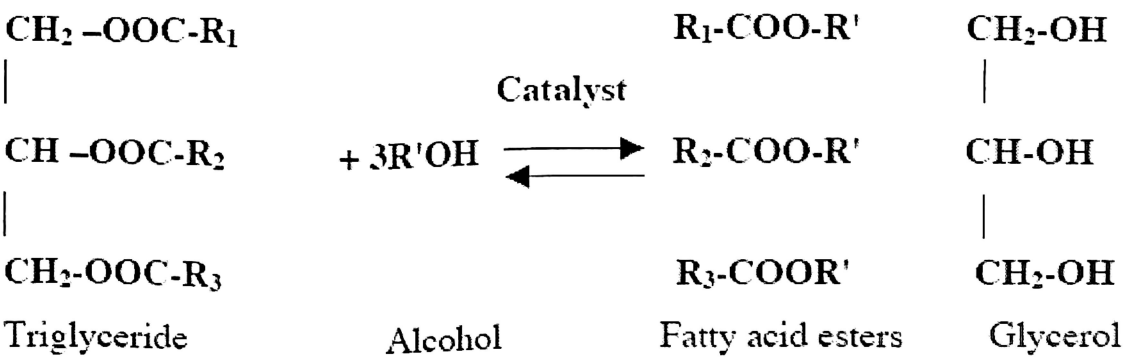
1.2 Biodiesel as an alternative fuel

In recent era, the escalating apprehension about the limited fossil fuel resources has led to the awareness that the amount of energy import should be decreased so as to become less dependent on oil exporting countries. Further, the impact of fossil fuel on the environment,

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especially the global warming and the harmful effects of carbon emissions, have created a new demand for clean and sustainable energy sources.

Biodiesel fuels have the potential to lower the net global warming gas emissions from the transportation sector and reduce the mass and carcinogenicity of particulate matter emissions. Biodiesel is the alkyl esters of fatty acids generally referred to as fatty acid alkyl esters (FAAE). Production of FAAE is achieved by the transesterification of animal fats or vegetable oils or the esterification of fatty acids, including free fatty acids (FFA) found in degraded fat or oil. Transesterification, also called alcoholysis, is the reaction of an oil or fat with an alcohol to form esters and glycerol. The reaction is shown in Scheme 1.1.



Scheme 1.1: Transesterification of triglycerides with alcohol

Esterification, transesterification, and hydrolysis can be carried out chemically or biocatalytically using enzymes. In the current industrial processes, homogeneous chemical catalyst, such as sodium hydroxide or sodium alkoxides are widely used. Although chemical esterification using the alkali-catalysts gives high yield of esters in short reaction times, the reaction has several drawbacks such as, energy intensive, difficulty in recovering the glycerol and catalyst from the reaction product due to soap formation, free fatty acids and moisture

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interference on the reaction. Moreover, the alkaline waste water generated from the downstream process requires treatment before being discharged to the environment. All these factors directly or indirectly affect the efficiency and economy of the process.

1.3 Lipase as a biocatalyst in biodiesel processing

With the growing concern and support on the green technology from government, private and other institutional bodies, the direction of the research is rapidly inclined towards the green chemistry-based process, resulting in growing research publications and patents on biocatalytic based production of biodiesel.

The activity of lipase, a well-known lipolytic enzyme, is not only confined to hydrolysis of fats, but is also extended to the biocatalysis of transesterification reaction. Under anhydrous condition, or in organic solvent lipase can catalyze transesterification; on the other hand, in aqueous solution, this enzyme catalyzes the hydrolysis reaction by splitting of triglycerides into fatty acids and glycerol. This versatility of lipase has realized its positive attribute on its potential application for the production of biodiesel for our future industries.

1.4 Techno-economic challenges of using lipase as catalyst

For industrial production of biodiesel, the downstream processing costs and environmental problems associated with biodiesel production and byproducts recovery, have led to the search for alternative production methods. Enzymatic transesterification using lipases has attracted attention for biodiesel production, as it produces high purity product and enables easy recovery of the byproduct glycerol, without any complex process. Moreover the free fatty acids contained in the oils can be completely converted to methyl esters and hence

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the subsequent wastewater treatment is not required. Lipase, a major lipolytic biocatalyst has some remarkable advantages like, energy-economic as the reaction could be carried out at room temperature, the presence of free fatty acids (FFAs) in the feedstock increases yield without soap production, thus creating opportunities for using a variety of lower-quality and hence lower-cost feedstock. Lipase works even in the presence of water and produces no salts. Considering the above facts, the lipase enzyme has been forecasted by the energy scientists as a potential catalyst for our future biodiesel industry.

Owing to their globular protein nature, lipases are spontaneously soluble in aqueous solutions whereas, their natural substrates (i.e. lipids) are not so. Therefore, to overcome the problem of poor intimate contact between substrate and enzymes, use of suitable organic solvent or emulsifier (like surfactant) in the reaction medium might be useful. However, the practical use of lipase in such pseudo-homogeneous reaction systems poses technological difficulties such as contamination of the products with residual enzymatic activity, and economic difficulties such as the use of enzyme in a single reactor pass. Hence, part of the overall potential of the enzyme is lost.

As a means of reducing the cost, the immobilization of microbial whole cell, as proposed by many researchers, skip the purification steps of the enzyme. While many advantages have been speculated, several serious disadvantages are also revealed on using whole cell biocatalysts such as, (i) development takes longer time due to complicated process caused by the need of elaborate experimental designs, which must take into account the cell growth with optimum catalytic activity (*i.e.*, enzyme manufacturing phase) and substrate conversion (*i.e.*, production phase) (ii) processes are messy if the cell broth is used directly, as the reactor may contains cells, and spent growth medium in addition to products and residual substrates that affects

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the downstream purification of the product-biodiesel, (iii) chance of producing unwanted by-products is high since many other enzymes, in addition to the desired lipase, may be present in the cells that may further degrade (oxidize or hydrolyze) the product biodiesel and (iv) slow kinetics of biodiesel production caused by the cell wall as diffusion barrier to the substrate from reaching the enzyme for catalysis. The vast majority of the lipase producing microorganisms secretes lipase to the growth medium and the molecular and functional characteristics of such secreted lipases from a wide variety of microorganisms are fairly understood. Moreover, unlike most of the enzymes and whole-cell biocatalysts, lipase can greatly withstand the effect of many organic solvent and hydrophobic environments –a property essential for a biocatalyst for its application to biodiesel production. However, for a continuous process, a soluble enzyme would be leached out from the reactor along with the product stream. A process like this would not be economically feasible as the biocatalyst is generally expensive. Although an ultrafiltration setup could be used to retain the enzyme, it is often too costly, both in capital and operation, on a large scale. The use of immobilized lipase could reduce the cost factor of the process due to easy regulation of activity, reusability, increased storage and thermal stability and the possibility of continuous operation. However, all these bright properties of an immobilized enzyme could be possible only when a suitable support matrix for its immobilization is identified and used. The support matrix must be biocompatible to maintain the activity of the native lipase under thermal and organic microenvironment, must cause less diffusion barrier to substrate and product, supports high reaction kinetics, and furnishes high surface area for high enzyme loading for the conversion.

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1.5 Scope of the work

Considering the enormous importance of immobilized lipase in the development of biodiesel production process, we direct our investigation to explore novel immobilizing support matrix for lipase, and utilize the immobilized lipase for the hydrolysis and transesterification of vegetable oil, with an ultimate aim to develop a biocatalytic process for the production of biodiesel.

1.6 Aim and objectives

In this dissertation, the aim is to study on immobilization of lipase and its application for biocatalytic hydrolysis and transesterification of vegetable oil for production of biodiesel.

The following objectives have been set to reach this goal: (a) Isolation, purification and characterization of microbial lipase. (b) Identification of a suitable matrix/material for immobilization of the lipase, (c) Application of the immobilized lipase for the hydrolysis of vegetable oil and (d) investigation on the immobilized-lipase catalyzed transesterification of vegetable oil for production of biodiesel in a suitable bioreactor.

1.7 Organization of the thesis

The study embodied in this thesis is categorized into the following chapters:

Chapter 1: Introduction

In this introductory chapter, a brief overview on the origin and importance of the work and objectives, with chapter wise division of the work done, is embodied.

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Chapter 2: Literature Review

The objective of this chapter is to summarize the progress of the work on the subject as a whole, focusing the gaps and important findings on the area. Current knowledge on the application of lipase enzymes in the hydrolysis and transesterification of vegetable oil is also presented here. A brief description on the sources of the lipase catalysts along with their properties with relevant literatures is also included in this chapter.

Chapter 3: Isolation, purification, production and characterization of a microbial lipase

This chapter describes the work starting from the isolation finally to the purification of a bacterial lipase. The screening of growth substrate and optimization of the medium component for production of lipase are also included here.

Chapter 4: Hydrolysis of vegetable oil using immobilized lipase

This chapter describes the immobilization of the lipase in few support materials- and application of the immobilized lipase for the hydrolysis of the vegetable oil. The immobilized lipase catalyzed hydrolysis of the oil was also investigated in presence of biosurfactant and details of the findings are reported here.

Chapter 5: Transesterification of vegetable oil using immobilized lipase for production of biodiesel

A packed-bed bioreactor for batch operation has been constructed using the lipase immobilized in a biomaterial and production of the biodiesel and byproduct glycerol was

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monitored in a time scale. A detailed account on this investigation is described in this chapter.

Chapter 6: Critical analysis and suggested future scope of the work

Critical analyses on the findings embodied in this thesis along with the scope for further research work are put forward in this concluding chapter.

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CHAPTER

LITERATURE REVIEW

2.1 Raw materials for biodiesel production

The use of vegetable oils in diesel engines is nearly as old as the diesel engine itself. The inventor of the diesel engine, Rudolf Diesel, reportedly used groundnut (peanut) oil as a fuel for demonstration purposes in the year 1900 (Nitske and Wilson, 1965; Shay, 1993). But it was immediately recognized that the direct use of vegetable oils led to carbon deposits and exhibited low pour-point problems. Because of these difficulties, biodiesel is used as fuel instead of using vegetable oil directly. Bruwer et al. (1980) reported that using an ester of sunflower oil (i.e. biodiesel) seemed to resolve the problems associated with the whole oil and in fact produced less carbon deposits in a test engine than petroleum derived diesel fuel. The raw materials usually being exploited for biodiesel production are the edible fatty oils derived from rapeseed, soybean, palm, sunflower, coconut, linseed etc. and non edible sources are rubber seed, tobacco seed, cotton seed, *Jatropha curcas, Pongamia pinnata, Mesua ferrea, Azadirecta species* etc. The main problem on commercial applications of biodiesel is the high cost of oils made from oleaginous crops. Recycled oil is one of the most promising alternatives in the production of biodiesel because of the low cost of the raw material and no expense of treating the oil as a residue.

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2.2 Biodiesel production

Among the several methods available for producing biodiesel, transesterification of natural oils and fats is currently the method of choice (Murugesan et al., 2009; Dennis et al., 2010; Karmakar et al., 2010; Mustafa and Havva, 2010; Lam et al., 2010). The brighter side of this approach is the low viscosity in the reaction mixture that helps manoeuvring the reaction and separation of the products. Transesterification using methanol as the co-substrate is the most commonly used method for production of biodiesel from triglyceride of vegetable oil or fat (Ma and Hanna, 1999; Srivastava and Prasad, 2000). This conversion was patented in the U.S. in 1950s, although the art of biolipid transesterification may have been discovered much earlier. There are numerous reports on the transesterification for biodiesel production (Trent, 1945; Tanaka et al., 1981; Allen et al. 1945; Freedman et al., 1984; Ali, 1995; Bradshaw and Meuly 1944; Ma et al. 1998; Ma et al. 1999; Antolin et al. 2002).

2.2.1 Biodiesel production through chemical transesterification

Currently, the alkali catalyzed transesterification of vegetable oil and animal fat is the most commonly used method (Gerpen et al., 1997; Ma et al., 1998; Wu. et al., 1999; Alcantara et al., 2000; Ikwuagwu et al., 2000; Chung et al., 2009; Sivakumar et al., 2011; Duz et al., 2011; Pinzi et al., 2011). The alkali catalysts include sodium hydroxide, potassium hydroxide, carbonates and corresponding sodium and potassium alkoxide such as, sodium methoxide, sodium ethoxide, sodium propoxide, and sodium butoxide. For alkali catalyzed transesterification, the triglycerides and alcohol must be substantially anhydrous because water causes a partial reaction change to saponification, which produces soap

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(Wright et al., 1944). The free fatty acid content of the refined oil should be below 0.5% (Ma et al., 1998).

Balasubramanian and Obbard (2011) investigated the role of both homogeneous (potassium hydroxide) and heterogeneous (calcium methoxide and calcium oxide) alkaline catalysts for transesterification of phosphatidylcholine (mixed with soybean oil in three different ratios i.e. 10%, 30% and 50%) to get biodiesel. According to their report, initial reaction rate is higher (yield 24.23 gram of ester per gram of catalyst per min) for homogeneous catalyst in comparison to its heterogeneous counterpart. Oguzhan (2011) studied the role of dolomite as catalyst for transesterification of canola oil with methanol to biodiesel in a heterogeneous system. He investigated the influence of the calcination temperature of the catalyst and the reaction variables. The maximum activity was obtained with the catalyst calcined at 850°C. When the reaction was carried out at reflux of methanol, with a 6:1 molar ratio of methanol to canola oil and a catalyst amount of 3% by weight, the highest ester yield of 91.78% was obtained after 3 hours of reaction time. Freedman et al. (1984) transesterified refined soybean oil with methanol using 1% NaOH catalyst at three different temperatures (60°C, 45°C, and 32°C) and the ester yields were found to be 94%, 87% and 64%, respectively. They compared both crude and refined vegetable oils as feed stocks of transesterfication and found that the methyl esters was reduced from 98% to 93% for refined oil, and to 86% to 67% for crude vegetable oil. This was attributed to the presence of free fatty acids in the crude oil. Kim et al. (2011) used strong alkaline phosphazenium hydroxide incorporated onto silica as a catalyst for methanolysis of vegetable (palm, corn, grape seed, and soybean) oils. Wright et al. (1944) reported that for an alkali-catalyzed

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transesterification the glycerides and alcohol must be substantially anhydrous, because water makes the reaction partially change to saponification.

Sivakumar et al. (2011) tried the alkali (1.2% KOH by weight of oil) catalyzed transesterification of dairy waste scum oil at 75°C, 350 rpm. 6:1 methanol oil ratio was identified to achieve a maximum bio-diesel yield of 96.7% in 30 minutes. MacLeod et al. (2008) reported the preparation of a series of alkali-doped metal oxide catalysts and evaluated them for activity in the transesterification of rapeseed oil to biodiesel. These catalysts appeared to be promising candidates to replace conventional homogeneous catalysts for biodiesel production as the reaction times were low enough to be practical in continuous process and the preparations were neither prohibitively difficult nor costly. However, metal leaching from the catalyst was detected, and this resulted in some homogeneous activity. Knothe (2001) performed experiment for production of biodiesel by reacting the oil or fat with an alcohol in the presence of basic catalyst to give the corresponding mono alkyl esters. Zamora et al. (1997) demonstrated a two-step laboratory method for transesterification of *Jatropha curcas* oil via alkali catalyzed methanolysis that yielded the ester concentration more than 99%. Foidl et al. (1996) prepared methyl and ethyl ester of *Jatropha* oil by transesterification process using potassium hydroxide catalyst, and found 92% yield and 88.4% yield for methyl and ethyl ester, respectively. They reported higher cost with ethanol process than that of methanol process. Raheman and Phadatre (2004) used alkali catalyst (potassium hydroxide) for transesterification of Karanja oil by methyl alcohol. Results show that final product is of such quality that it can be used in village machinery without further treatment. Similar result obtained by Kumar et al. (2003) for production of biodiesel from *Jatropha* oil. Zuraida and Hameed (2011) used calcium oxide as a solid base supported in

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carbon for methanolysis of cooking grade of palm oil. Catalyst was found to sustain after two cycles of transesterification. They got 80.98% yield of methyl ester which was well within their predicted model according to their central composite design based optimization via Response surface methodology. The parameters they optimized were reaction time (1 hour 21 minutes), molar ratio of methanol to oil (15:1), reaction temperature (190°C) and catalyst amount (5.5 % by weight).

Mao et al. (1998), Boocock (2001) and Zhou et al. (2003) developed a novel technique for accelerating the transesterfication reaction rate. During its early stages, transesterification reaction was limited by the low solubility of the alcohol in oil. But Boocock proposed the addition of a co solvent to create a single phase that accelerates the reaction and complete it in a few minutes. Ma et al. (1998) studied the effect of reaction time on transesterification of beef tallow with methanol and observed that the reaction was very slow during the first minute. The reaction proceeded quickly from 1 to 5 minutes. Freedman et al. (1984) transesterified peanut, cottonseed, soybean and sunflower oils under the condition of methanol to oil ratio of 6:1, 0.5% NaOMe catalyst and 60°C. They achieved 80% of yield of ester after 1 minute, for soybean and sunflower oil, but after 1 hour the conversions were observed to be 93-98% for all the oils.

Duz et al. (2011) demonstrated microwave irradiation of the crude sunflower oil with methanol at a molar ratio of 10:1, in the presence of 1.0% NaOH catalyst as a better and faster (6 min over 98.4% yield of methyl ester) technique in comparison to conventional heating during alkali catalyzed alcoholysis of triglycerides with methanol. The effect of alcohol to oil ratio on the yield of the ester was studied by several researchers. Bradshaw and Meuly (1944) reported that the practical range of molar ratio was from 3.3 to 5.25:1 of

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vegetable oils (Jatropha and Mahua) and the effect of co-solvent on this transesterification reaction. According to their observation, the use of tetrahydrofuran (THF) as a co-solvent enhances the reaction rate. If that reaction being modeled as a single step reaction then, it is second order with respect to triglyceride concentration and is first order with respect to methanol concentration. Kinetic study of base catalyzed methanolysis of monoglyceride (obtained from low-cost Pongamia oil) using alkaline caustic potash performed by Karmee et al.(2004) using proton NMR revealed the improvement of transport effect with the gradual increase in temperature from 45 to 60°C. Therefore, the forward reaction rate constant of this reversible second order reaction found to increase with the increase in temperature. Karmee and Chadha (2005) prepared biodiesel from crude Pongamia pinnata oil and methanol (1:10 molar ratio) at 60°C using inorganic catalysts (caustic potash, Hβ-Zeolite, montmorillonite K-10 and ZnO) . Addition of co-solvent Tetrahydrofuran (THF) helped to improve the maximum conversion of oil to ester from 92% to 95% when caustic potash was used as a catalyst. Viscosity (4.8 Cst @ 40 °C) and Flash point (150°C) of methyl ester were found to be comparable with ASTM and German biodiesel standards. Crude Pongamia oil has viscosity of 74.14 Cst @ 30 °C. Later, Karmee et al. (2006) studied the kinetics of three-step methanolysis of Pongamia oil at 60°, by calculating the forward and reverse reaction rate constants. According to their observation, the forward rate reaction constant is gradually decreased in the subsequent stages from triglyceride (TG) to diglyceride (DG) and from DG to monoglyceride (MG).

Pinzi et al. (2011) reported that the biodiesel yield and economic viability of the process depends upon fatty acid composition and the optimal reaction temperature. Ma et al. (1998) studied the effects of FFAs and water on transesterification of beef tallow and

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methanol to vegetable oil. Higher molar ratios result in greater ester conversion in a short time. In the ethanolysis of peanut oil, a 6:1 molar ratio liberated more glycerin than did a 3:1 molar ratio (Feuge and Grose, 1949). Nye and Southwell (1983) methanolysed rapeseed oil using 1% NaOH or KOH and found that, a molar ratio of 6:1 of methanol to oil achieved the best conversion. Freedman et al. (1984) applied a molar ratio of 6:1 of alcohol to oil in transesterification of soybean, sunflower, peanut and cottonseed oils and achieved a very high conversion (93-98%). Ali (1995) and Zhang (1994) also used a molar ratio of 6:1 for beef tallow transesterification. Avellaneda and Salvadó (2011) developed a continuous transesterification by integrating batch reactors in a single line using a device consisting of helicoidal tube through which reactants flow. They claimed to reduce the reaction time from 75 minutes (batch process) to 13 minutes (in their continuous process of capacity 2.5 ml/min) with 89% ester yield.

Reaction kinetics of alkali-catalyzed transesterification is largely affected by the chemical and physical properties of the oils. Bradshaw and Meuly (1944) and Feuge and Grose (1949) stressed the importance of oils being dry and free from fatty acids. The alkali catalyzed conversion is complicated if oil contains higher amounts of free fatty acid (FFA) (>1% w/w) that generates soap with alkaline catalyst. The soap can prevent separation of the biodiesel from the glycerin fraction. To avoid the effect of saponification Canakci and Gerpen (1999) worked on acid catalyzed production of biodiesel. Keim (1945) demonstrated that acid catalysed transesterification can be used if triglycerides contain more water and free fatty acids. Sprules and Price (1950) also reported that when large amount of FFA was present in the oil, a molar ratio of 15:1 was needed during acid catalysis. Ravi Kumar et al. (2011) did kinetic study on alkali (1% KOH) catalyzed methanolysis of two non-edible

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reported that FFAs content should be kept below 0.06% w/w in order to get the best conversion. Dorado et al. (2002) used feedstocks bearing higher FFA levels. They used pretreatment step to reduce the FFAs of these feedstocks to less than 1% before being used the feedstock in the transesterification reaction catalyzed by alkali. They optimized the pretreatment process to reduce the FFA content of mahua oil below 1% for maximum biodiesel production. Ginosar et al. (2007) patented a relatively unique process of producing an alkyl ester using gas expanded alcohol and a triglyceride or fatty acid. Reed et al. (1992) reported a method for producing esters from waste cooking oils containing significant quantities of FFA liberated during the cooking process. They suggested addition of caustic soda to the waste cooking oils to neutralize the FFAs by converting them to soap prior to transesterification. Freedman et al. (1984) reported that alkali-catalyzed transesterification is faster than acid-catalyzed transesterification. However, they noted that glyceride containing higher FFA and water is suitable for acid-catalyzed transesterification. Further they also observed that sodium methoxide is a better catalyst than sodium hydroxide.

Tanaka et al. (1981) developed a novel method of preparing methyl esters of fatty acids glycerides contain shorter alkyl group. Zhang (1994) transesterified beef tallow with a free fatty acids content of 0.27%. In this process the tallow was heated to remove moisture under vacuum, and then it was kept at 60°C. The transesterification reaction was conducted using 6:1 molar ratio of methanol to tallow and 1% (w/w by the weight of tallow) sodium hydroxide dissolved in the methanol and 60°C for about 30 minutes. Following the separation of glycerol, the ester layer was transesterified again using 0.2% NaOH and 20% methanol at 60°C for about 1h. The yield of ester was 80%. Wimmer (1992) prepared methyl esters by esterification of glycerides with C 1-5 alkanols C 2-5 alkoxy alkanols in the presence of basic

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catalysts. Stern et al. (1995) patented a method for making fatty acid esters from acid oils. In his method initially the FFAs in the oil were allowed to react with glycerol to form glycerides, which was then added to the next alcoholysis step. Ikwuagwu et al. (2000) reported the production of methyl ester using rubber seed oil and sodium hydroxide as catalyst. Alcantara et al. (2000) described the catalytic production of biodiesel from soybean oil, used frying oil and tallow by transesterification and amidation reactions with methanol and diethylamine, respectively.

The acid catalysts usually include sulphuric acids, sulphonic acids and hydrochloric acids. Haas et al. (2002) (2003) have shown that acid-catalyzed esterification can be used to produce biodiesel from low-grade by-products of the oil refining industries, such as soapstock. Soapstock is a mixture of water, soap and oil, was dried, saponified and then esterified with methanol or some other simple alcohol using an inorganic acid as catalyst. Zhang et al. (2003) reviewed the commonly used procedures for producing biodiesel. They reported that use of waste cooking oil feedstocks provided a higher rate of return than refined vegetable oils even after including the additional capital and operating costs of acid catalyzed pretreatment. The preferred conditions for acid-catalysed esterification of carboxylic acids or transesterification of existing esters, are therefore a large excess of the appropriate alcohol and the absence of water. While it may be possible to obtain water-free conditions simply by adding anhydrous sodium sulfate to the reaction medium (Molnar and Pinter, 1986), a better practice in general is to operate with dry reagents and glassware. A critical practical point is the choice of acid as catalyst. This must facilitate the reaction but should not cause unwanted side effects. In principle, the methodology can be used with any alcohol component, but in

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practice it is limited to those alcohols that can be eliminated from the reaction medium by selective evaporation.

Sprules and Price (1950) used an alkali and then acid-catalyzed transesterification process for high acid value oils. According to a recent kinetic study (Jain et al., 2011) of acid-base [1%(w/w) H_2SO_4 and NaOH] catalyzed two step transesterification of pretreated waste cooking oil, transesterification (at 50°C) was found to be faster (rate constant 0.0078 min^{-1}) than esterification (65°C) (rate constant of 0.0031 min^{-1}). In their experiment, complete conversion of triglyceride to methyl ester took 6 hours of time with maximum yield of methyl ester 21.50% in case of esterification and 90.6% in case of transesterification. Fang Yan et al. (2010) have prepared a solid Fe (II)-Zn double-metal cyanide complex. They observed that this solid has a highly catalytic activity to carry out the simultaneous transesterification of triglycerides and esterification of free fatty acids with 98% yield of methyl ester. After 6 cycles of reaction, 93.45% catalyst was found to be recovered. Even at higher content of water or free fatty acids this catalyst has exhibited its high activity. Kumar and Ali (2010) have applied nanocrystalline (particle size of about 50 nm) lithium carbonate impregnated CaO as a heterogeneous catalyst for transesterification of used cottonseed oil with a higher amount of moisture (up to 15% by weight) to produce methyl ester.

Free fatty acids contained in oils and fats could also be converted efficiently to methyl esters in supercritical methanol. Giridhar et al. (2004) gave the details of such procedure for synthesis of biodiesel. According to Saka and Kusdiana (2001), methanol and rapeseed oil, in a molar ratio of 42:1, if preheated to a temperature of 350°C for 240 seconds in supercritical methanol, then sufficient methyl esters would be produced. Barnwal and Sharma (2004) gave theoretical knowledge of both catalyzed and supercritical method of

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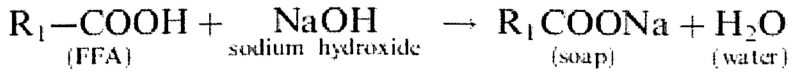
transesterification process to produce biodiesel. According to them, catalyzed process is easy but supercritical method gives better result. Noh et al. (2007) patented a method for producing biodiesel in the form fatty acid alkyl ester by esterifying oils and fats, even from waste vegetable oil or animal fat by supercritical fluid for producing alkyl ester of high purity at low cost and high productivity. Supercritical fluid technology possesses a number of advantages that includes fast kinetics, high fuel production rate, and ease of continuous operation and elimination of the necessity of catalysts. However, the harsh operation environment (i.e. the high temperature and high pressure) and high materials cost are the main concerns for its wider application. The main focus on state-of-the-art application of this technique includes biodiesel from vegetable oils via the transesterification process, bio-hydrogen from the gasification and bio-oil from the liquefaction of biomass (Dongsheng et al., 2009).

2.2.2 Biodiesel production through enzymatic transesterification

Chemical esterification using an alkali-catalysis process although gives high yield of esters in short reaction time, the reaction has several drawbacks: it is energy intensive, recovery of the byproduct glycerol is difficult, the catalyst has to be removed from the product, free fatty acids and moisture interfere with the reaction, and alkaline waste water requires treatment before discharging to the environment (Shah et al., 2004). As a specific case to cite the disadvantage of alkali-catalyzed transesterification process, if the feedstock contains high percentage of free fatty acid or water, it may lead to some undesirable side reactions, where the alkali instead of acting as catalyst, may directly participate in the reaction. For example, the catalyst alkali reacts with the free fatty acid present in the

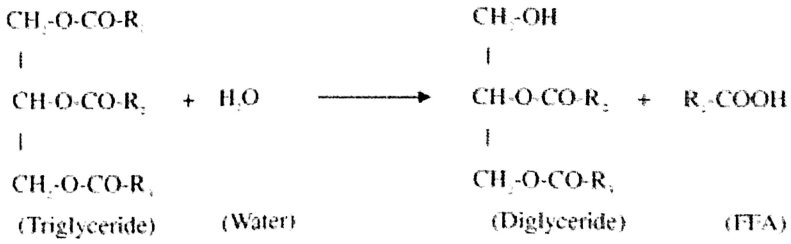
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substrate oil to form soap. This kind of acid alkali neutralization phenomenon is well-known as saponification reaction.



Scheme 2.1: Saponification reaction

The water on the other hand, can hydrolyze the triglyceride to form more amount of FFA.



Scheme 2.2: Hydrolysis of triglyceride

The aforementioned side reactions have negative consequences on ester yield and thus are undesirable for the biodiesel production. To get rid of the difficulties involved in alkali catalyzed and acid catalyzed transesterification different alternative strategies have been proposed, among which enzymatic transesterification methods have received much attention since last one and half decades (Fukuda et al., 2001; Yu-Yen et al., 1998; Bajaj et al., 2010; Mittelbach, 1996). The enzyme mostly being used for such transesterification and hydrolysis of lipid for biodiesel production is lipase. The lipase catalyzed transesterification is superior to chemical transesterification, for biodiesel preparation on several points such as, lack of soap formation, mostly green technology, as the reaction could be carried out at room temperature without using any chlorinated or toxic solvents, easy to separate the product from the byproduct glycerol etc. However, the stumbling block for commercialization of this process is the high cost of production and purification of the enzyme lipase and low operational stability of the enzyme catalyst (Huang and Yan, 2008). Although the use of

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lipases makes the reaction less sensitive to high free fatty acid content which is a problem with the standard biodiesel process, the methanol may inactivate the lipase catalyst after initial batch. However, if methyl acetate is used instead of methanol, the lipase is not in-activated and can be used for several batches, making the lipase system much more cost effective (Du et al.; 2004). Based on the large volume of literature cited in this chapter, a general comparison among the various methods used for biodiesel production is given in Table 2.1.

Table 2.1: Advantages and limitations of different catalysts used in transesterification reaction for biodiesel production

Catalyst type	Example	Advantage	Disadvantages
Alkali: Homogeneous	NaOH, KOH	High catalytic activity, low cost, favorable kinetics, modest operation conditions	High moisture and free fatty acid content cause saponification, emulsion formation, Disposable problem of alkaline wastewater.
Alkali: Heterogeneous	CaO, $CaTiO_3$, $CaZrO_3$, CaO–CeO_2, $CaMnO_3$, $Ca_2Fe_2O_5$, KOH/Al_2O_3, KOH/NaY, Al_2O_3/KI, ETS-10 zeolite, alumina/silica supported K_2CO_3	Noncorrosive, environmentally benign, recyclable, fewer disposal problems, easily separation, higher selectivity, longer catalyst lifetimes	Requirement of anhydrous conditions and low free fatty acids; disposable problem of alkaline wastewater; requirement of high molar ratio of alcohol to oil, high reaction temperature and pressure, diffusion limitations, high cost.
Acid: Homogeneous	Concentrated sulphuric acid	Catalyze esterification and transesterification simultaneously, avoid soap formation	Equipment corrosion; more waste from neutralization; difficult to recycle; higher reaction temperature; long reaction times; weak catalytic activity.
Acid: Heterogeneous	Carbon-based solid acid , carbohydrate-derived , vanadyl phosphate, niobic acid, sulphated zirconia, Amberlyst-15, Nafion-NR50.	Catalyze esterification and transesterification simultaneously, recyclable, eco-friendly.	Low acid site concentrations; low microporosity; cause diffusion limitations; high cost.
Enzymes	Microbial Lipases from *Candida Antarctica*, *Rhizomucor miehei*	Avoid soap formation, ecofriendly, easier purification	Expensive; low operational stability due to denaturation.

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The lipase production cost however, has been reduced substantially due to the advent of advanced genetic engineering techniques and identification of new sources for high and stable lipase, since the last few decades. The hyper lipase producing microbial strain could be developed with high levels of expression and/or stability towards methanol (Casimir et al., 2007). Lipase (triacyl glycerol ester hydrolase, EC 3.1.1.3) is an enzyme that catalyzes the hydrolysis of triacylglycerols to fatty acids, mono- and di-acylglycerols, and glycerol. It also catalyzes the reverse reactions, such as esterification, inter-esterification, and transesterification. Lipase is one of the most versatile and widely used enzymes in biotechnological applications due to its unique properties (Salameh and Wiegel, 2007).

Lipases are widely distributed in plants, animals, insects and microorganisms (Gilbert, 1993; Hasan et al., 2009; Maya and Diana, 2011). Activity of this enzyme has been identified in various parts of plants, animals and insects, but relatively higher concentration is found in the seeds. About 60% of this enzyme is associated with glycosomes, 15% with mitochondria, and remaining 25% with a membrane fraction in some oil-seeds. (Pahoja and Sethar, 2002). In animal, the lipases are found in pancreas, and on the surface of mucous cells of the gastric mucosa. The enzyme is found mostly in plasma, salivary glands, muscles and fat bodies in insect. Microorganisms produce both intracellular and extracellular lipases. Among the lipases from various sources the microbial lipases find immense application. This is because microbes can be easily cultivated and their lipases can catalyze a wide variety of reactions. A brief general comparison on the molecular mass, optimum pH and temperature, activator and inhibitor of the lipases among the aforesaid three different sources are given in Table 2.2.

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Table 2.2: A broad characteristics of lipases from different sources:

Lipase origin	Animal	Plant	Microbes
Molecular weight range (KDa)	43 to 300	19 to 270	32 to 97
Optimum temperature range (°C)	37 to 60	20 to 38	30 to 75
Optimum pH range	4.0 to 9.0	5.5 to 8.5	6.0 to 10.0
Activator	Ca^{++}, Zn^{++}	Ca^{++}, Zn^{++}	Surfactants, sugar ester
Inhibitor	EDTA, Triton X100, Tween 80	EDTA, Triton X100, Tween 80	Fe^{+++}, Zn^{++}, Hg^{++}

The lipases can be measured and purified by titrimetric, colorimetric, fluorometric, thin layer chromatography, gas chromatography, ammonium sulphate precipitation, Sephadex G-75 and DEAE-cellulose chromatography (Saxena et al., 2003).

Enzymatic transesterification using lipases has attracted attention for biodiesel production as it produces high purity product and enables easy recovery from the byproduct, glycerol without any complex process. The use of immobilized lipases and immobilized whole cells may lower the overall cost, while presenting less downstream processing problems, to biodiesel production (Man et al., 2009). Moreover the free fatty acids contained in the oils can be completely converted to methyl esters and subsequent wastewater treatment is not required. Owing to their globular protein nature, lipases are spontaneously soluble in aqueous solutions whereas, their natural substrates (i.e. lipids) are not so. Therefore, to overcome the problem of poor contact between substrate and enzyme suitable organic solvent or an emulsifier (like surfactant) might be useful. However, the practical use of lipase in such

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pseudo-homogeneous reaction systems pose technological difficulties such as, contamination of the products with residual enzymatic activity, and economic difficulties such as, the use of enzyme in a single reactor pass. Hence, part of the overall potential enzymatic activity is lost. But if the lipase is immobilized, then it becomes an independent phase within the reaction system, which may easily be retained in the reactor with associated advantages in preventing contamination of the products and extending its useful active life. Further, increasing the temperature generally increases the rate of lipase-catalyzed reaction per unit amount of active enzyme. However, increasing the temperature also leads to a higher thermal deactivation rate of the lipase (Katarzyna and Krzysztof, 2009; Marilyne and Didier, 2003). Because immobilization provides a more rigid external backbone for lipase molecule, temperature optima are expected to increase, which results in a faster reaction rate. Another challenge associated with the lipase catalyzed alcoholysis of fats is that lipases can be deactivated by lower linear alcohols, such as methanol and ethanol, conventionally used in biodiesel process (Maceiras et al., 2011). However, if methyl acetate is used instead of methanol, the lipase inactivation could be reduced (Du et al., 2004). Although the enzymatic production of biodiesel has been proposed to overcome the drawbacks of the conventional chemically catalyzed processes, the main impediment facing complete exploitation of the potential of lipase is its cost. Therefore, from the economic point of view, the reuse of this enzyme is essential, which can be achieved by using the lipase in immobilized form. In addition, the immobilized lipase displays improved stability and activity. Mostly practiced immobilization techniques include entrapment within the matrix of a polymer and attachment to solid supports (Noureddini et al., 2005; Chen and Lin, 2003; Vaidya et al., 2008; Chaubey et al., 2009; Fabiano et al., 2010; Rafael and Roberto, 2010).

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The activity and stability of enzyme depends largely on the particular operating and storage conditions and are strongly influenced by some factors such as temperature pH, solvent property, chemical environment etc. (Mattias et al., 2002; Katarzyna and Krzysztof, 2009). Most of the enzymes are soluble in water and so for their solubilization certain amount of water is always essential. This factor should be taken care of during application of immobilized enzyme for some practical reactions. During immobilization, the biocatalyst (enzyme) restrained or localized in a microenvironment yet retains its catalytic property. Immobilization habitually increases stability and makes the reuse of the enzyme preparation very simple. This frequent analysis that can be performed by the employment of enzymes in immobilized form reduces the cost of the analysis. The perfect immobilization practice for a given enzyme permits a high turnover rate of the enzyme yet retains a high catalytic activity over time (Suckling, 1977).

Different microbial lipases, such as *Candida Antarctica*, *Pseudomonas cepacia* and *Thermomyces lanuginosus* have been extensively used as biocatalysts. Both intracellular and extracellular lipases are reported to be effective in catalyzing the transesterification of triglyceride in either aqueous or non-aqueous systems (Bisen et al., 2010; Helwani et al., 2009). In such systems the byproduct glycerol can be easily recovered without any complex process and also the free fatty acids, contained in waste oils and fats can be completely converted to methyl esters. A comparative account on the yield of ester using lipase from various sources has been compiled with some selected references as given in Table 2.3.

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Table-2.3: Enzymatic transesterification reactions using various types of lipases.

Lipase Source	Oil used	Acyl-acceptor	Solvent	Maximum yield (%)	Reference
B. subtilis 1.198	Waste cooking oil	Methanol	NIL	90	Ying and Chen, 2007
B. cepacia (free enzyme)	Jatropha oil	Ethanol	NIL	79	Shah and Gupta, 2008
C. rugosa	Rapeseed oil	2-ethyl-1-hexanol	NIL	97	Linko et al., 1998
C. antarctica	Cottonseed oil	Methanol	t-butanol	97	Royon et al., 2007
	Mixture of soybean and rapeseed oils	Methanol	NIL	98.4	Shimada et al., 1999
	Fish oil	Ethanol	NIL	100	Breivik et al., 1997
	Sunflower oil	Methanol	NIL	97	Belafi-Bako et al., 2002
Candida sp.	Salad oil	Methanol	NIL	96	Nie et al., 2006
	Frying oils	Methanol	NIL	92	Nie et al., 2006
	Microalgae	Methanol	Hexane	98	Li et al., 2007
Different lipases *[M. miehei, C antarctica, G. candidum, R. delemar,* and *P. capacica]*	Tallow	Different primary alcohols	Hexane	94.8-98.5	Nelson et al., 1996
	Soybean oil	Different secondary alcohols	Hexane	61.2-83.8	Nelson et al., 1996
Commercial enzyme preparation from a recombinant *Aspergillus* strain expressing *T. lanuginosus* lipase	Palm oil	Ethanol	*tert*-butanol	-	Raita et al., 2010

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Lipase Source	Oil used	Acyl-acceptor	Solvent	Maximum yield (%)	Reference
C. viscosum	Jatropha oil	Ethanol	NIL	92	Shah et al., 2004
E. aerogenes	Jatropha oil	Methanol	t-butanol	94	Kumari et al., 2009
Geotrichum sp.	waste cooking oil	Methanol	Different polar and non-polar organic solvents	83	Jinyong Yan et al., 2011
P. capacica	Soybean oil	Methanol	NIL	67	Noureddini et al., 2005
	Soybean oil	Ethanol	NIL	65	Noureddini et al., 2005
M. miehei	Rapeseed oil	Methanol	t-butanol	95	Li et al., 2006
	High oleic sunflower oil	Butanol	n-Hexane	95	Dossat et al., 2002
M. miehei, *C. antarctica*	Sunflower oil	Methanol	NIL	-	Türkan and Kalay, 2006
C. antarctica (Novozym 435), *M. meihei* (Lipozyme TL IM)	Soybean oil deodorizer distillate (28% FFA)	Methanol	*t*-butanol	Around 95	Wang et al., 2006
	Waste edible oil (2.5% FFA)	Methanol	NIL	>90	Watanabe et al., 2001; Shimada et al., 2002
M. meihei(Lypozyme)	Sunflower oil	Ethanol	NIL	83	Selmi and Thomas, 1998

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Lipase Source	Oil used	Acyl-acceptor	Solvent	Maximum yield (%)	Reference
Novozym 435 (*C. antarctica* lipase immobilized acrylic resin)	Acidic oil (77.9% FFA)	Methanol	NIL	>90	Watanabe et al., 2007
	Soybean (crude, degummed and refined) oil	Methanol	NIL	93.8	Watanabe et al.,2002
	Sunflower oil	Methanol	NIL	3	Mittelbach, 1990
	Sunflower oil	Ethanol	NIL	82	Mittelbach, 1990
	Jatropha, Sunflower	2-propanol	Hexane	92.8-93.4	Modi et al., 2006
	Jatropha, Sunflower	Ethyl acetate	NIL	91.3	Modi et al., 2007
	Rapeseed oil	Methanol	t-butanol	95	Li et al., 2006
Novozym 435 (*C. antarctica* lipase immobilized acrylic resin)	Soybean oil	Methyl acetate	NIL	92	Du et al., 2004
	Soybean oil	Methanol	NIL	97	Samukawa et al., 2000
P. cepacia	Palm kernel oil	Methanol	NIL	15	Abigor et al., 2000
	Palm kernel oil	Ethanol	NIL	72	Abigor et al., 2000
	Waste cooking oil	Methanol	NIL		Sulaiman Al-Zuhair et al., 2009
P. cepacia (Lipase PS-30) + *C. Antarctica* (Lipase SP-435)	Recycle Resturant grease	Ethanol	NIL	85.4	Wu et al., 1999
Powdered lipase QLM	Phytosterol containing (10%) sunflower oil	Phytosterol	NIL	97.1	Negishi et al., 2003
P. cepacia (lipase PS 30)	Soybean oil	Methanol	NIL	67	Noureddini et al., 2005

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Lipase Source	Oil used	Acyl-acceptor	Solvent	Maximum yield (%)	Reference
P. fluorescens	Sunflower oil	Methanol	NIL	90	Soumanou and Bornscheuer, 2003
	Soybean oil	Ethanol	Iso-octane	71	Zhao et al., 2007
	Soybean oil	Methanol	*n*-Heptane	92	Yu et al., 2006
P. fluorescens/ *R. miehei* (Novozym-435)	Sunflower oil	Methanol	Petroleum ether	79	Mittelbach, 1990
R. miehei	Sunflower oil	Methanol	*n*-Hexane and Petroleum ether	80	Soumanou and Bornscheuer, 2003
R. miehei *H. isolens*	Sunflower oil	Methanol	Water	91.2	Oliveira and Rosa, 2006
R. miehei (*Lipozyme IM-77*)	Soybean oil	Methanol	*n*-Hexane solvent	92.2	Shieh et al., 2003
R. oryzae	Soybean oil	Methanol	NIL (Water content 4-30% by weight)	80-90	Kaieda et al., 1999
R. mucilagenosa P11I89	Refined palm oil	Methanol	NIL	83.29	Purimprat et al., 2011
T. lanuginose	Soybean oil	Methanol	NIL	-	Du et al., 2004
	Pomace oil	Methanol (in three steps)	NIL	93	Yücel, 2011
T. lanuginosus and *P. fluorescens*	Palm oil and babassu oil	Ethanol	NIL	-	Adriano et al., 2011
Various commercial lipases	Microalgae	Long-chain alcohols	Hexane	-	Ramírez et al., 2006
Various commercial lipases [lipase B from *Candida antarctica*, Lipozyme RMIM(from *Rhizomucor miehei*), PS-C and *PS-D* (from *P. cepacia*)]	Triolein	Mixtures of linear and branched alcohols	NIL	~ 100	Salis et al., 2005

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Nelson et al. (1996), Domingos et al. (2008), Ranganathan et al. (2008) and Antczak et al. (2009) demonstrated the enzymatic production of biodiesel. Research also studied the lipase catalyzed transesterification conditions. These include study on identifying the appropriate lipase, purification of the enzyme through modern efficient techniques such as, expanded bed chromatography, affinity precipitation and three phase partitioning (Vasudevan, 2005). Linko et al. (1998) demonstrated the production of a variety of esters and polyesters with lipase as the biocatalyst. Lipases are known to have a propensity to act on long-chain fatty alcohols better than short-chain ones (Shimada et al., 1999). Brask et al. (2011) proposed a combined method of lipase (Novozym 435) catalyzed esterification of high free fatty acid contained oil followed by alkali catalyzed transesterification in an integrated approach in packed bed reactor. Shah et al. (2004) reported that lipase from *Chromobacterium viscosum* immobilized on Celite-545 can catalyze the transesterification of *Jatrapha Curcus* oil to ensure highest yield of ethyl ester compared to the lipases from other two sources (*Candida rugosa,* and *porcine pancreas)*. Yagiz et al. (2007) used waste oils for lipase catalyzed transesterification. In their work they used hydrotalcite and four different types of zeolites used as immobilization material. Tan et al. (2010) reviewed the application of immobilized lipase for the production of biodiesel. They reported immobilization of *Candida antartica* lipase on acrylic resin and immobilization of *Candida* sp. 99–125 lipase on inexpensive textile membranes. Gray et al. (1990) immobilized lipase from *Candida cylindraceae* on EP-400 polyethylene by the cellulose-titanium chloride method to carry out transesterification. Yilmaz et al. (2011) evaluated the enzymatic property of immobilized *Candida rugosa* lipase on magnetic sol–gel composite supports through model hydrolysis of p-nitrophenylpalmitate. They observed that the sporopollenin based

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encapsulated lipase particularly has higher conversion and enantioselectivity compared to the encapsulated lipase without supports. Liu et al. (2011) studied the influence of magnetic field on the properties of magnetic nanoparticles using SEM, XRD and VSM. They observed that magnetic field enhanced the activity, operational and storage stability of the immobilized lipase. For ethanolysis of different feedstocks (mainly babassu oil and tallow beef), Patricia et al.(2010) immobilized *Burkholderia cepacia* lipase (lipase PS) on two different non-commercial matrices [inorganic matrix (niobium oxide, Nb_2O_5) and a hybrid matrix (polysiloxane–polyvinyl alcohol, SiO_2–PVA)] by covalent binding. They got distinct biochemical properties, kinetic constants and thermal stability during the use of SiO_2–PVA. Martin and Otero (2008) made a comparative study on two different enzymes [Novozym® 435 and Lipozyme® TL IM] for synthesis of biodiesel. The *R. Oryzae* lipase that exhibits 1(3)-regiospecificity was also found to be effective for the methanolysis of soybean oil as demonstrated by different groups (Okumura et al., 1976; Macrae, 1983; Matori et al., 1991). In the transesterification of rapeseed oil with 2-ethyl-1-hexanol 97% conversion of esters was obtained using *candida rugosa* lipase powder. Abigor et al. (2000) found that in the conversion of Palm oil to alkyl esters using *P. cepacia* lipase, ethanol gave the conversion of 72% while only 15% methyl esters was obtained with methanol. Kaieda et al. (2001) investigated the methanolysis of soybean oil with both non-regiospecific and 1(3)-regiospecific lipases in a water containing system without an organic solvent. Li et al. (2006) used a novel organic solvent for lipase catalyzed production of rapeseed oil ester. Xu et al. (2005) developed a new enzymatic approach using methyl acetate as a novel acyl acceptor for production of biodiesel from soybean oil. They found the highest yield (92%) of methyl ester on using immobilized *Candida antarctica* lipase (Novozym 435) as catalyst. They also

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optimised the reaction temperature (40°C), reaction time (10 hours), molar ratio of methyl acetate/oil (12:1) and enzyme concentration (30% enzyme based on oil weight). Since no glycerol was produced in the process, this method is very convenient for recycling the catalyst, and byproduct triacetylglycerol showed no negative effect on the fuel property. Royon et al. (2007) carried out the methanolysis of cottonseed oil using immobilized *Candida antarctica* lipase as catalyst in *t*-butanol solvent. They monitored the chemical conversion from triglyceride to alkyl ester by HPLC. The enzyme inhibition which may happen on using methanol was overcame by adding t-butanol into the reaction media ; which further increased the reaction rate and ester yield. Modi et al. (2006) shown that propan-2-ol can be used as accyl acceptor for lipase catalysed transesterification. They suggested that propan-2-ol is better acyl acceptor than methanol or ethanol. They found that the reusability of this enzyme was intact even after 12 repeated cycles while using propan-2-ol, whereas, in case of methanol, after the 7^{th} cycle, it reached to zero under standard reaction conditions. Türkan and Kalay (2006) observed that lipozymes RM IM and TL IM catalyze the first step faster, whereas Novozym 435 catalyzes the second and third steps faster. So they suggested a dual enzymatic system (e.g., Novo 435/TL IM) rather than single lipase systems for production of biodiesel from vegetable oils. The ethanolysis of tuna oil in two (or three) reaction steps, as well as in a single-step, have been investigated as means for obtaining enhanced conversions (Watanabe et al., 1999). Iso et al. (2001) used lipase catalyzed transesterification reaction, using vegetable oils and short chain alcohol. They used immobilized lipase in non-aqua's condition. Immobilization of lipase was carried out using porous kaolinite particle as carrier. Result shows that immobilization of lipase gives better result. Gray et al. (1990) immobilized lipase from *Candida cylindraceae* on EP-400

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polyethylene by the cellulose-titanium chloride method to carry out transesterification. Brask et al. (2011) suggested an integrated biodiesel process that combines alkaline transesterification followed by enzymatic esterification (enzymatically converted to fatty acid methyl esters) of palm fatty acid distillate obtained from palm oil refining plant, in a two-step process using the immobilized lipase Novozym 435 (*Candida antarctica* lipase immobilized acrylic resin), in packed-bed columns. Fukuda et al. (2001) optimized the biocatalysis of transesterification reaction by using super critical fluids, and they established that this enzymatic approach can be a better proposition over the conventional thermo-chemical means. Mittelbach (1990) shown that lipase catalyzed transesterification is a most viable method for the production of alkyl esters from oil. Bélafi-Bakó et al. (2002) studied methanolysis (transesterification with methanol) of sunflower oil by lipase from *Candida antarctica* (Novozym 435) in a solvent-free system. They applied stepwise as well as continuous methanol feeding. Glycerol was removed by dialysis using a flat sheet membrane module as it may cause strong product inhibition on the enzymatic reaction. Pizarro et al. (2003) reported the lipase-catalyzed production of biodiesel fuel from vegetable oil which was derived from the waste of crude vegetable oil. Methanolysis was efficiently catalyzed by lipase in the presence of high water content and by a single addition of methanol. The highest conversion yield reached 55% (w/w) with palm oil after 96 hours of reaction. Adverse viscosity condition might have influenced methanolysis of extracted soybean and rapeseed oil in spite of high water and methanol concentration. Shah et al. (2004) reported a lipase catalyzed tranesterification procedure for *Jatrapha Curcus* oil. In their work three different lipases (*Chromo bacterium viscosum*, *Candida rugosa,* and *porcine pancreas*) were screened for a transesterification reaction of Jatropha oil in a solvent-free system to produce biodiesel.

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Only lipase from *Chromobacterium viscosum* was found to give appreciable yield. Immobilization of lipase (*Chromobacterium viscosum*) on Celite-545 enhanced the biodiesel yield to 71% from 62% yield obtained by using free tuned enzyme preparation with a process time of 8 hours at 40°C. Further, addition of water to the free (1%, w/v) and immobilized (0.5%, w/v) enzyme preparations enhanced the yields to 73% and 92%, respectively. Immobilized *Chromo bacterium viscosum* lipase can be used for ethanolysis of oil. It was seen that immobilization of lipases and optimization of transesterification conditions resulted in adequate yield of biodiesel in the case of the enzyme-based process. Raita et al. (2010) developed an economical heterogeneous biocatalyst, by protein-coated microcrystals (in *tert*-butanol system), from a commercial enzyme preparation from a recombinant *Aspergillus* strain expressing *Thermomyces lanuginosus* lipase, for the biocatalytic ethanolysis of palm oil. Fukuda et al. (2008) performed a comparative study on immobilized and whole cell commercial lipases as a biocatalyst for enzymatic production of biodiesel from Jatropha oil. Aleksandra et al. (2011) used exopolysaccharide produced by an extremophylic strain of *Pseudomonas aeruginosa san-ai* as a support for lipase immobilization. They have successfully reused their preparation up to 3 repeated cycles with a residual activity of 77% of the initial was retained afterwards. Some review reports (Sarma et al., 2008; Robles-Medina et al., 2009; Tianwei et al., 2010; Semwal et al., 2011; Joon et al., 2011) described abundant literatures are available on enzymatic transesterification. Jackson and King (1996) employed an immobilized lipase to catalyze the methanolysis of corn oil in flowing superficial carbondioxide with an ester conversion of more than 98%. Nadir et al. (2009a) used *Thermomyces lanuginosus* lipase covalently immobilized in microporous polymeric matrix (containing as aldehyde functional group; synthesized by polyHIPE technique using

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styrene, divinylbenzene, and polyglutaraldehyde), to carry out transesterification of various vegetable oils (sunflower, soybean, and waste cooking oils) at 25°C for biodiesel synthesis in accordance with design of experiment based on Taguchi methodology. They successfully carried out 10 repeated batch reaction of 24 hours each with complete retention of enzymatic activity. Moreno-Pirajàn and Giraldo (2011) used immobilized *Candida rugosa* lipase in activated carbon support for transesterification of palm oil with different straight chain alcohols and they evaluated the production yield of biodiesel using flow microcalorimetry. Earlier most of the researchers had used mainly oils from the source like soybean, rapeseed, sunflower and safflower that are essentially edible in nature. Nye (1984) attempted for producing methyl ester from frying oil. Wu et al. (1999) optimized the production of ethyl esters of grease using 95% ethanol by response surface methodology. According to their finding, time and temperature have significant effect whereas the lipase level had the modest effect on response. Tianwei et al. (2010) reviewed the application of immobilized lipase for the production of biodiesel. According to their report, two most commonly available techniques are immobilization of *Candida antartica* lipase acrylic resin and *Candida* sp. 99–125 lipase on inexpensive textile membranes. Bruno et al. (2004) immobilized *Mucor miehei* lipase on magnetic polysiloxane-polyvinyl alcohol particles by covalent binding. Robles-Medina et al. (2009) suggested for paying particular attention to the use of whole cell immobilization in the production process as this methodology may reduce both the cost of the biocatalyst and dependence on manufacturers. Aleksandra et al. (2011) used exopolysaccharide as a support for immobilization of *Pseudomonas aeruginosa* lipase. Both the lipase and exopolysaccharide were produced during cultivation of the microorganism in Luria Bertani (LB) media with sunflower oil and Tween80. They also prepared calcium-

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exopolysaccharide beads by dripping a concentrated culture supernatant in calcium- chloride solution to entrap lipase. According to their report, this type of immobilized enzyme preparation could be used up to three repeated cycles to retain a residual activity of 77%. Iso et al. (2001) also used porous kaolinite particle as carrier to immobilize lipase in non-aqueous condition. Usai et al. (2010) immobilized lipase from *Candida Antarctica.* Cléber et al. (2011) used MCM 22 zeolite as support material for immobilization of porcine pancreatic lipase. They observed the highest yield and lipase activity at Si/Al ratio of 25 among three (15, 25 and 50) different such ratios. Elif et al. (2011a) used chemically inert sol–gel support (prepared by polycondensation with tetraethoxysilane and octyltriethoxysilane in the presence and absence of magnetic Fe_3O_4 nanoparticles or sporopollenin with Fe_3O_4 as additive) for encapsulation of *Candida rugosa* lipase. Anna et al. (2011) recently studied the effect of various parameters [surface coverage at different enzyme-Celite ratios and the effect of molar ratio of different alkylTEOS (alkyl = propyl, hexyl, octyl, 1*H*,1*H*,2*H*,2*H*-perfluorooctyl, decyl, dodecyl, octadecyl), PhTEOS and TEOS (seven series of alkylTEOS:PhTEOS:TEOS from 0.1:0.9:1 to 0.9:0.1:1 in 0.1 steps) on the enzymatic properties] on *Pseudomonas fluorencens* lipase supported on Celite via sol–gel immobilization using ternary silane precursor systems having alkyltriethoxysilane (alkylTEOS), phenyltriethoxysilane (PhTEOS) and tetraethoxysilane (TEOS). They got the best overall result with the medium-chain octylTEOS and perfluorooctylTEOS-containing ternary systems. Elif et al. (2011) immobilized *Candida rugosa* lipase (CRL) on aminopropyl glass beads after activating with glutaraldehyde using covalent binding method or sol–gel encapsulation procedure and improved considerably by fluoride-catalyzed hydrolysis of mixtures of $RSi(OCH_3)_3$ and $Si(OCH_3)_4$. Adriano et al. (2011) used Toyopearl AF-amino-

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650M resin for covalent attachment of two different microbial (*Thermomyces lanuginosus* and *Pseudomonas fluorescens*) lipases simultaneously with different activating agents, mainly glutaraldehyde, glycidol and epichlorohydrin. Their immobilized enzyme preparation was useful to carry out transesterification of babassu and palm oils with ethanol in solvent-free media. They got highest hydrolytic activity and thermal stability, between 27 and 31 times more stable than the soluble lipase. Monier et al. (2010) modified natural wool fibers by means of graft copolymerization of poly ethylacrylate in presence of potassium persulphate and Mohr's salt redox initiator to immobilize *Candida rugosa* lipase. In their work, under the optimum condition of 40°C and pH 8.0, the relative activity of immobilized lipase was found to be higher in comparison to free sample (at pH 7.5). Also the immobilized samples were found to be more stable at a wider pH range. The kinetic constant value (K_m) of immobilized lipase was higher than that of the free lipase. However, the thermal and operational stabilities of immobilized lipase were improved greatly. Nadir et al. (2009) reported the immobilization of *Thermomyces lanuginosus* lipase using both physical adsorption and covalent attachment against polyglutaraldehyde activated styrene–divinylbenzene copolymer.

The lipase is a lipolytic enzyme that functions at oil-water interface and generates less undesirable by products during hydrolysis of fats and, hence, its application potential as catalysts for hydrolysis of oil and fat is also increasingly focused (Linfield et al., 1984; Park et al., 1988; Moussata and Akoh, 1998; Zhao et al., 2007). Since interfacial activation is essential for catalytic activity of lipase (Zaks and Klibanov, 1985), various surface-active agents are employed to emulsify the fat in aqueous phase for enhancing the hydrolytic activity of lipases. However, majority of the surface-active agents utilized for the hydrolysis

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are synthetic and non-biodegradable in nature. Thus, the effluent generated from the down-stream processing may cause environmental pollution if preventive measure is not taken. Biosurfactants are usually biodegradable surface active agents. One of the focuses of our work is also to utilize biosurfactant for the lipase-catalyzed hydrolysis of vegetable oil. Hydrolysis of fats and oils is also a well-known important activity in oleo chemical industries for the production of fatty acid, which is a renewable feedstock for industrial-scale processing to give value-added products for food, beverages, cosmetics and medicinal applications (Hasan et al., 2006, Gandhi, 1997). In the fatty acid industry, hydrolysis of fats and oils is usually accomplished by a high-temperature steam treatment method that operates with super-heated steam and high pressure (Brady et al., 1988). This high-temperature process is energy intensive, and causes extensive degradation of the produced fatty acids. There is a growing interest to replace this chemical method with enzymatic methods as the later methods are normally operated under mild conditions in an energy efficient manner. Lipase catalyzed hydrolysis can overcome the problems of the earlier industrially implemented Colgate-Emery method (Kent; 1974) for the hydrolysis of triglycerides, where the process utilized steam of high-temperature (523 K) and high-pressure (5.00×106 Pa), results in high energy consumption and thermal damage of the products (Villeneuve et al., 2000; Xu, 2003). The advantage of enzymatic hydrolysis is the mild reaction conditions. Moreover, the enzymes have substrate and positional specificities (Villeneuve et al., 1995; Benjamin and Pandey; 1998; Gunstone, 1999; Xu, 2000; Sharma et al., 2001; Muralidhar et al., 2001) and therefore the side reactions such as, saponification, polymerization and oxidation could be prevented to enhance the yield of the desired product. In the triglyceride

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hydrolysis using lipase, the substrates are oil and water, and the hydrolysis takes place at the oil-water interface.

Covalent immobilization of enzyme on the support matrix is most commonly practiced technique till date. However, the binding reaction must be performed in such a way so that the active site of the enzyme should not be affected by the reagents being used and the reaction process should not affect the enzyme activity. Sometimes it is possible to increase the number of reactive residues of an enzyme for increasing the yield of insolubilized enzyme and to provide alternative reaction sites to those essential for enzymatic activity. Renny et al. (2008) reported the use of covalently immobilized lipase from *Pseudomonas cepacia* on semi-conducting crystalline/porous silicon and silicon nitride surfaces to develop biosensor. Nadir et al. (2009) used *Thermomyces lanuginosus* lipase covalently immobilized in microporous polymeric matrix (containing as aldehyde functional group; synthesized by polyHIPE technique using styrene, divinylbenzene, and polyglutaraldehyde) to carry out transesterification of various vegetable oils (sunflower, soybean, and waste cooking oils) at 25°C for biodiesel synthesis following the design of experiment based on Taguchi methodology. They have successfully carried out 10 repeated batch reaction of 24 hours each with complete retention of enzymatic activity. In case of successful cross-linking, covalent bonding should provide stable, insolubilized enzyme derivatives that do not percolate enzyme into the surrounding solution. The wide variety of binding reactions, and insoluble carriers with functional groups capable of covalent coupling, or being activated to give such groups, makes this a generally applicable method of insolubilization, even if very little is known about the protein structure or active site of the enzyme to be coupled. Yücel (2011) immobilized *Thermomyces lanuginosus* lipase by covalent binding onto olive pomace with

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maximum lipase loading density of 18.67 mg/g support and the highest specific activity of 10.31 U/mg protein to retain its activity even after ten repeated batch of transesterification of pomace oil using methanol with a maximum biodiesel yield of 93% (at 25°C in 24 hours). Since last few years, magnetic nano-particles have been identified as a promising technology for immobilization of enzyme by covalent bonding (Ponvel et al., 2009; Dussan et al., 2010; Wenlei and Ning, 2010; Bruno et al., 2010; Liu et al., 2011).

Enzyme can be immobilized by intermolecular cross-linking, either to other protein molecules or to functional groups on any insoluble support matrix. Self cross-linking of any enzyme is expensive; because some of the protein material will unavoidably be acting mainly as a support, which results relatively low activity of the enzyme. To overcome the drawback of deactivation of the enzyme, Rios et al. (2011) used different acidic resins (viz. Amberlite IR-120, Dowex 50WX2, Nafion-silica) and immobilized lipase as heterogeneous catalysts for the epoxidation of jatropha oil. Among these resins Amberlite IR-120 was the best catalyst, yielding 70% epoxide selectivity and was stable through five re-uses with a high level of cross-linking to achieve as high as 90% biodiesel yield. According to their finding, low-accessible acid sites increase epoxide selectivity. Glycol selectivity could be increased by highly accessible acid sites and acid strength favors the rate of epoxidation. Generally, cross-linking is best used in conjunction with one of the other methods. Jinyong et al. (2011) observed the better improvement of thermal stability, organic solvent tolerance, pH stability and operational stability of *Geotrichum* sp. lipase by double modifications during immobilization of the lipase (cross-linking and protein coating with K_2SO_4).

Lee et al. (2006) immobilized *Rhyzppus oryzae* lipase in silica gel by glutaraldehyde cross linking. They have optimized the different stages of immobilization procedure like,

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pretreatment of silica gel, salinization, cross-linking, glutaraldehyde modification, and the elimination of unreacted aldehyde groups. According to their observation, 80% of the enzymatic activity was retained even after 20 consecutive reuse of the immobilized enzyme. Chiou et al. (2007) used Genipin, (a reagent of plant origin) for immobilization of lipase by cross-linking to chitosan beads. In their observation, about 74% of the initial enzyme activity was found to be retained after five cycles of hydrolysis. Because of mass transfer limitations they have observed a lower energy of activation and a high apparent K_m value.

Lipases are water-soluble enzymes that catalyze the hydrolysis of triacylglycerols and a large variety of natural and unnatural esters. They differ from one another in their physical properties and biochemical features and are selective in respect to the fatty-acid chains. The hydrolysis of ester bond catalyzed by lipase involves acylation and deacylation steps (Grochulski et al., 1994, Malcata et al., 1992). The substrate selectivity in the hydrolysis reaction is believed to be determined by the tetrahedral intermediate formed in the acylation step. Lipase catalyzed transesterification of triglycerides with an alcohol (alcoholysis) involves a two-step mechanism when looking at a single ester bond (Scheme 2.4). The first step is hydrolysis of the ester bond and release of the alcohol moiety followed by an esterification with the second substrate (Kaieda et al., 2001; Zhou et al., 2001). The two steps are represented by the following two equations as shown in scheme 2.3 (Paiva et al., 2000).

$$E + Es_s \leftrightarrow E \,.\, Es_s \leftrightarrow F \,.\, B_p \leftrightarrow F + B_p$$

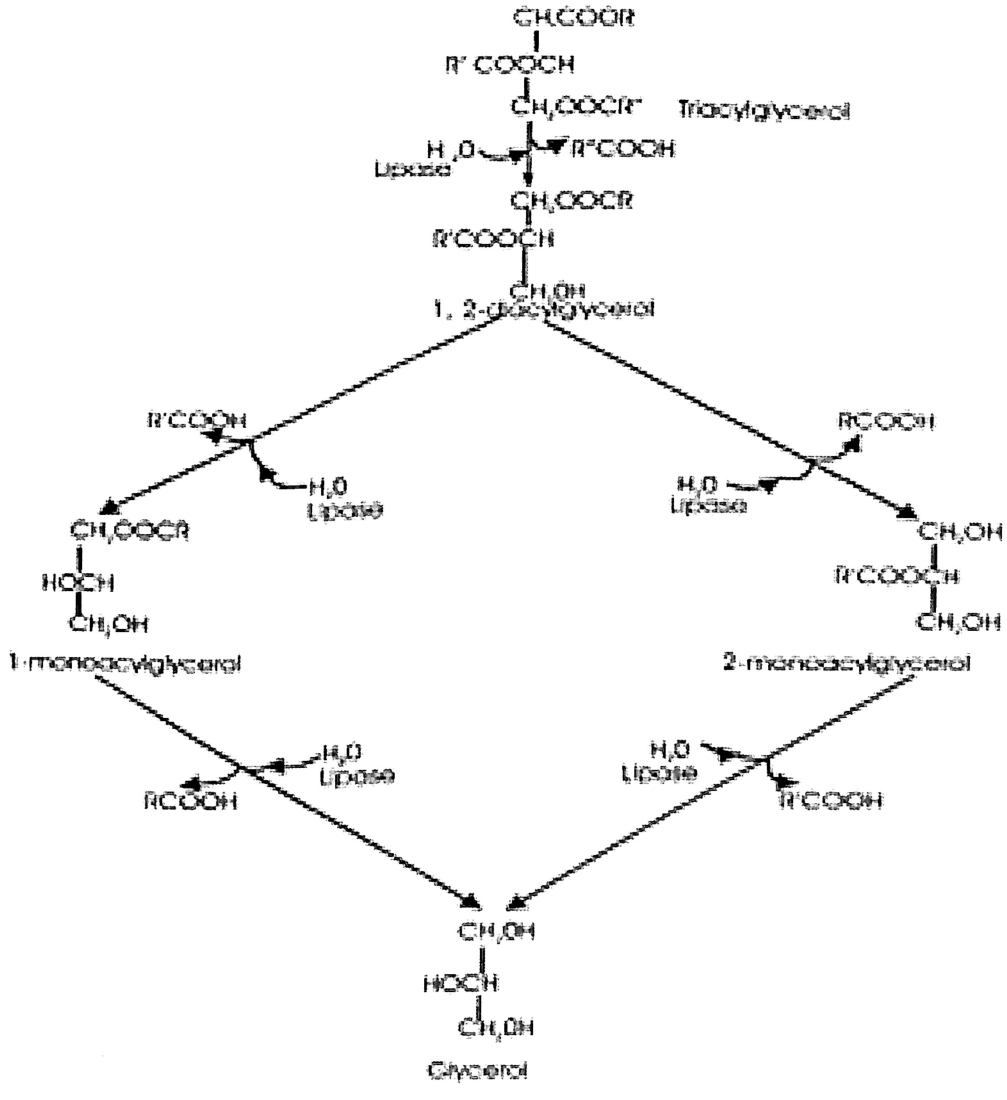
Followed by

$$F + A_s \leftrightarrow F \,.\, A_s \leftrightarrow E \,.\, Es_p \leftrightarrow E + Es_p$$

Scheme 2.3: Lipase catalyzed esterification

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Where, the subscripts's' and 'p' indicate substrate and product, respectively. For biodiesel, A_s = alcohol substrate (i.e., methanol or ethanol), B_p= product with alcohol moiety (di- or monoglyceride or glycerol), E = free enzyme, Es_s = ester substrate (tri-, di- or monoglyceride), Es_p = Fatty acid alkyl ester, F = fatty acid.



Scheme 2.4: Reaction mechanism of lipase (Source: Pahoja and Sethar, 2002)

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Isolation, purification, production, and characterization of a microbial lipase

3.1 Overview

Lipases may be of plant, animal, or microbial origin. Among these sources, microbes can be easily cultivated to produce lipase in a large quantity. Therefore in practice, microbial lipases are widely used for lipase-based biocatalytic processes. Till date different microbial lipases such as, *Thermomyces laguginosus* (Fernandez-Lafuente, 2010), *Candida antarctica* (Usai et al., 2010), *Rhizopus oryzae* (Ting et al., 2010) *Pseudomonas cepacia* (Sakai et al., 2010) are employed as biocatalyst for biotransformation of lipid. The commercially purified lipases are expensive. Moreover, the information on many commercially available lipases such as, their origin, activity, stability, specificity and other substantial details are not available and the reason being attributed is to their trade tactics in some cases. Hence, exploration of lipase from indigenous source and their characterizations for further applications has been urged in this investigation. This chapter describes the isolation, purification, and characterization of a lipase from a bacterial strain originally isolated from a petroleum hydrocarbon contaminated soil. The screening of substrate for the production of this extracellular lipase from the bacterial strain has also been carried out to identify a suitable substrate for the lipase production. Detail reports on these investigations are incorporated in this chapter.

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3.2 Experimental approach

3.2.1 Materials

Pseudomonas agar, *n*-Hexadecane, *n*-octadecane, glycerol tributyrin, glucose, DEAE sepharose, sodium chloride, BSA, sodium phosphate, chicken ovalbumin, chicken egg-albumin, chymotrypsin, sodium hydroxide, phenolphthalein were of reagent grade and bought from M/S AB Chemical (India). Polyurethane foam and sunflower oil was collected from the local market. Burnt oil was collected from the I.C. Engine testing rig in Centre for Energy, IIT Guwahati. All other chemicals and reagents were of analytical grade and were used as received, without any modification.

3.2.2 Cultivation of the organism and culture conditions

A lipase producing *Pseudomonas* strain, originally isolated from oil-contaminated soil, used in this investigation was taken from the stock culture of the Biofuel cell research laboratory, Centre for Energy, IIT Guwahati. The organism was cultured in 250 ml Erlenmeyer flask containing 2% *n*-hexadecane as sole source of carbon for energy and growth in mineral media. Mineral media composition (in 1 liter distilled water): KNO_3----4g; Na_2HPO_4----3.61g; KH_2PO_4----1.75g; $MgSO_4 \cdot 7H_2O$---0.2g; $CaCl_2 \cdot 2H_2O$----0.05g; $FeSO_4 \cdot 7H_2O$----1mg; $CuSO_4 \cdot 5H_2O$----50µg; MoO_3----10µg; $MnSO_4$----10µg. pH was adjusted at 6.8 by using 1M HCl. The flasks are agitated at 200 rpm at 32°C for growth of the organism. The organism was maintained in *Pseudomonas* agar slant at 30°C.

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3.2.3 Identification of the microorganism

The pure bacterial strain in a culture tube was sent to Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India, for identification. The identification was done following the molecular analysis, using the standard PCR protocol for detecting *Pseudomonas* species by partial 16S ribosomal DNA sequencing (Widmer et al., 1998; Boye et al., 1999). Genomic DNA was first isolated from the bacteria followed by amplification of 16S rDNA region by using universal primers: 27 forward (5′AGAGTTTGATCMTGGCTCAG3′) and 1492 reverse (5′GGTACCTTGTTACGACTT3′). The amplification cycle consisted of an initial denaturation step of 5 minutes at 94°C, followed by 29 cycles of 1 minute at 94°C, 1 minute at 48°C and 2 min at 72°C and a final extension of 10 minutes at 72°C. Sequencing of the amplified product was done by dideoxy chain terminator method using the Big Dye terminator kit followed by capillary electrophoresis on an ABI 310 genetic analyzer (Applied Biosystems, USA). The sequence obtained was BLAST searched and compared with sequences of other closely related species retrieved from the Gene Bank database of IMTECH, Chandigarh, India.

3.2.4 Assay of lipase

Lipase activity was determined by glyceryl tributyrin-polyvinyl alcohol emulsion method, which is a partial modification of the method described by Ibrahim et al. (1987). 1% (w/v) polyvinyl alcohol dissolved in 50 ml water by heating at 50°C to 60°C. 2% (v/v) glyceryl tributyrin was dissolved in the aforementioned solution and sonicated to form an

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emulsion. 4 ml of this emulsion solution was mixed with 5 ml solution of sodium phosphate buffer (0.1N) pH 7.0 and 1 ml of enzyme solution (or 0.5 mg lipase) in a 250 ml of conical flask and incubate for 20 minutes in an orbital shaker at 30°C at 200 rpm. The reaction was terminated by adding 20 ml solution of acetone: ethanol at the ratio 1:1. Free fatty acid liberated was titrated against 0.02 N sodium hydroxide solution using phenolphthalein as indicator. One unit of lipase activity was defined as the amount of enzyme that liberates one micromole of free fatty acid per minute at 30°C.

3.2.5 Isolation of lipase

Culture broth obtained by cultivating the *Pseudomonas* strain as per the procedure stated above was harvested at 36 hours. The harvesting time of 36 hours for the strain was chosen based on an earlier work reported by Kanwar et al. (2002) that the growth of a *Pseudomonas* strain on alkane substrate in similar media composition falls in the range of late exponential phase but not in the stationary phase. The harvesting at 36 hours thus nullifies the possible complexity being aroused by the cell lysis on the isolation of the lipase from the culture broth. The broth was then centrifuged (Beckman Ultracentrifuge, USA) at 10,000×g for 20 minutes. The supernatant obtained was then filtered through Millipore membrane filter of size 0.45 μm, to remove bacterial cells and other particulate impurities.

A floating protein layer containing lipase activity was fractionated from the cell free supernatant using ammonium sulfate at a saturation of 60% (w/v). The enzyme layer was separated at 10000 ×g and re-suspended in a minimum volume (10-13 ml) of 20 mM sodium phosphate buffer pH 7.0, and then desalted by dialyzing for 4 hours against the same buffer (500 ml × 2). The desalted lipase solution was further concentrated by lyphylization

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(Micromodulyo Freeze Dryer, make: Thermo Electron Corporation, model: Micromodulyo 0230). The dry lipase powder obtained by lyophilization was assayed for the activity and kept at 4°C for further use.

3.2.6 Purification of Lipase by ion exchange chromatography

The cell free supernatant obtained from the culture broth as described in section 3.2.5 was dialyzed overnight at 4°C in 500 ml of 20 mM sodium phosphate buffer pH 7.0. The dialized supernatent was lyophilized to obtain a salt free lipase powder. A suitable quantity of the powder was solubilized in 20 mM sodium phosphate buffer, pH 7.0, and filtered through 0.45 µm Millipore filters prior to loading on the column. A DEAE sepharose column was equilibrated with 20 mM sodium phosphate buffer, pH 7.0, and lipase protein was loaded on the column at a flow rate of 1ml/min. The elution of the adsorbed protein was done at a flow rate of 5ml/min with a linear gradient of 0-200 mM NaCl in 20 mM sodium phosphate buffer, pH 7.0. The eluted fractions (2ml each) were collected in HPLC fraction collector (DuoFlow, Biologic DuoFlow QuadTec, Bio-Rad). The fractions containing lipase activity were pooled and then concentrated by lyophilization. The lipase obtained by ammonium sulfate precipitation (at 60% cut-off) was also dissolved in a suitable volume of 20 mM sodium phosphate buffer, pH 7.0, and purified by ion exchange chromatography following similar procedure as stated above. The purified dry powdered sample was stored at 4°C for further use.

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3.2.7 SDS-PAGE analysis

Molecular weight of the purified lipase was determined by SDS-PAGE following the standard protocol of Laemmli (1970) using 5% stacking and 10% separating gel. As per standard protocol, to make 10ml of a 5% (stacking) polyacrylamide gel, 1.65ml 30% acrylamide, 2.5ml 1.0 M Tris (pH 6.8), 0.1ml 10% SDS, 0.1ml 10% ammonium persulfate, and 0.004ml TEMED (tetramethylethylenediamine) were dissolved in 5.65ml double distilled water. Similarly, to make 10 ml of a 10% (resolving) polyacrylamide gel, 3.3ml 30% acrylamide, 2.5 ml 1.5M Tris (pH 8.8), 0.1 ml 10% SDS, 0.1 ml 10% ammonium persulfate , and 0.004 ml TEMED (tetramethylethylenediamine) were dissolved in 4.0 ml double distilled water. Gel size 3"×5"×0.2", each takes roughly 8 ml of resolving and 2 ml of stacking gel. The gel was run (miniVE, vertical electrophoresis system, Amarsham Biosciences, USA) at constant 30 mA until the dye front (Bromophenol blue) reached the bottom of the gel. The gel was stained with Coomassie Brilliant Blue (CBB) R-250 to visualize the protein bands. Destaining was done with a mixture of acetic acid, and methanol (4:1 v/v). The standard protein markers used for relative comparison of the molecular weight were BSA (66 KDa), Chicken Ovalbumin (46 KDa), Chicken Egg-albumin (35 KDa), Chymotrypsin (29 KDa).

3.2.8 Estimation of protein

Protein was estimated by Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard. Coomassie Brilliant Blue (CBB) G-250 was used which is

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known to bind with amino acids content in the protein giving a maximum absorption in the region of 595 nm wavelength.

3.2.9 Temperature optima, pH optima, and thermal stability of the isolated lipase

To measure pH optima, the enzyme reactions were carried out in different pH buffers namely, trisodium citrate buffer (pH 4), sodium acetate buffer (pH 5), sodium phosphate buffer (pH 6 and 7), tris buffer (pH 8), ethanolamine buffer (pH 9), and piperazine buffer (pH 10), each at a concentration of 50 mM. For pH stability measurement, the enzyme samples were incubated for different time periods in the aforementioned pH buffers at 4°C and the residual catalytic activity was determined at 30°C in sodium phosphate buffer (50 mM, pH 7.2). The optimum temperature was determined by carrying out the enzyme reactions at different temperatures and the catalytic activity was determined by the method described in section 3.2.4. For determination of temperature stability, enzyme samples were incubated at different temperatures (20°C to 80°C) in circulating water bath (Julabo SW22, Germany) for different time periods followed by measurement of residual activity 30°C in sodium phosphate buffer (50 mM, pH 7.2). The $t_{½}$ (half-life) was calculated from the value of 0.693/k, where 'k' is the first order rate constant. The value of 'k' was calculated from the slope of a curve obtained by plotting $\ln(a_0/a_t)$ vs time (t), where a_0 is the initial activity and a_t is the residual activity obtained after incubating the enzyme for different time periods at the said pH or temperature.

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3.2.10 Statistical analysis

All the data presented in this study were the mean of three or more experiments with a variation within 10%. Analysis of variance (ANOVA) test was carried out for the statistical data, at p-value < 0.05 with the help of standard method of experimental uncertainty analysis (Kline and McClintok, 1953). When the experimental value was found to be significant, critical difference was calculated by multiplying the standard error of difference with the corresponding error values at 5% level of probability. Significant differences among the means were determined by using least significant difference test.

3.3 Results and Discussion

3.3.1 Optimization of the media for production of lipase

The *Pseudomonas* strain identified as *Pseudomonas aeruginosa* PG01, following 16s ribosomal DNA sequencing, was cultivated using *n*-hexadecane as substrate in the mineral media as stated in the experimental section. Various nitrogen and phosphate compounds were screened for the production of lipase using *n*-hexadecane as the sole carbon substrate. Following composition and concentration of the constituents in the basal medium was found to be optimum for the production of lipase (in 1 liter distilled water): KNO_3----2g; $(NH_4)_2SO_4$----2g; Na_2HPO_4----3.61g; KH_2PO_4----1.75g; $MgSO_4 \cdot 7H_2O$----0.2g; $CaCl_2 \cdot 2H_2O$----0.05g; $FeSO_4 \cdot 7H_2O$----1mg; $CuSO_4 \cdot 5H_2O$----50µg; MoO_3----10µg; $MnSO_4$----10µg. The pH value of the solution was adjusted to 6.8.

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3.3.2 Screening of substrate for lipase production

Various hydrophobic as well as amphipathic substrates (2 g/l) were studied separately for the production of lipase by the *P. aeruginosa* PG01 in the defined mineral media composition stated elsewhere. Highest lipase activity in the culture supernatant was found when sunflower oil was used as the sole substrate as shown in Table 3.1. A significant production of lipase in burnt oil was also observed, which was about 75% of the activity obtained when sunflower oil was used as the substrate. Lack of activity in glucose substrate may be attributed to the role of glucose as catabolic repressor [Stülke and Hillen (1999)].

Table 3.1: Screening substrates for the production of lipase by *P. aeruginosa* PG01

Sl. No.	Substrate(2g/l)	Lipase activity (U/ml)
1.	Glucose	0.00
2.	n-Hexadecane	3.9±0.20
3.	n-Octadecane	3.5±0.16
4.	Sunflower oil	4.1±0.05
5.	Burnt Mobil oil	3.1±0.20
6.	n-Glycerol tributyrin	2.0±0.08
7.	n-Glycerol tributyrin + n-Hexadecane	3.4±0.30

Each value represents the mean ± standard error at $p < 0.05$

Long chain hydrocarbon as sole carbon substrate for microbial production of lipase has been reported in the laboratory (Kanwar et al., 2002). However, the burnt mobil as sole

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carbon substrate for production of microbial lipase revealed from this investigation is new and rated potential for contributing economic viability of lipase catalyst based processes.

3.3.3 Purification of the lipase

The ion exchange chromatogram on the purification of the lipase is shown in Figure 3.2. The cell-free supernatant containing lipase with specific activity of 8.1 U/mg-protein was adsorbed in DEAE sepharose column. The lipase was desorbed from the column at a sodium chloride concentration of 21 mS. The specific activity of lipase in the desorbed fraction was 21 U/mg-protein. Hence the lipase was purified to 2.59 fold (Table 3.2). The lipase solution from the purified fraction when pooled and concentrated by lyophilization, the lipase activity in the lyophilized powder was found to be 15.8±0.20 U/mg-protein. The result showed partial denaturization of the lipase after lyophilization. The reason is ascribed to the extensive dehydration of the lipase powder occurred during lyophilization that likely to affect the structural integrity of the lipase protein.

Table 3.2: Purification of *P. aeruginosa* PG01 lipase by DEAE-sepharose column chromatography

Sl. No.	Fractions	Enzyme activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg-protein)	Fold purification
1.	Cell free extract	2.1±0.20	0.10±0.04	8.1±0.01	1
2.	Lipase in the eluted fraction	2.4±0.22	0.29±0.02	21.0±0.03	2.59

Each value represents the mean ± standard error at $p < 0.05$.

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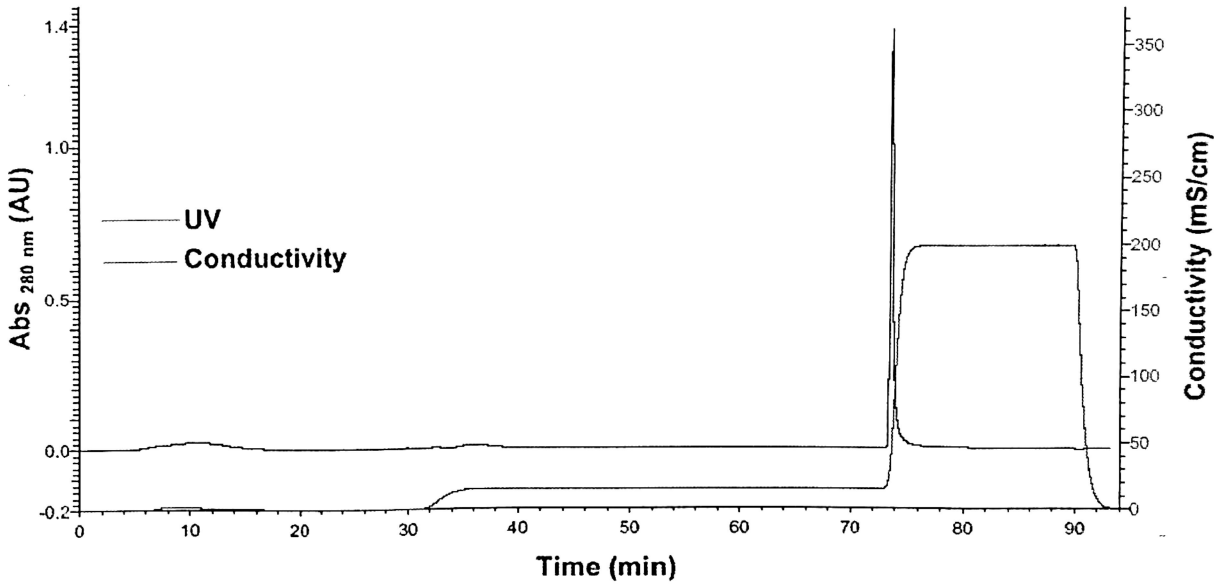


Figure 3.1: Ion exchange chromatogram of the lipase using DEAE sepharose column

The synthetic medium used in the present investigation does not contain any proteinaceous compounds and complex substrate in the culture supernatant that reduces the chance of secretion of other enzymes bearing substrate metabolic liabilities, which is the likely reason for only marginal increase of specific activity in the ion exchange purified fraction of the lipase.

3.3.4 Molecular weight of the lipase

The homogeneity of the DEAE purified protein was demonstrated by single protein band in native PAGE. The molecular weight of the purified lipase protein was estimated by SDS-PAGE and was found to be nearly equal to 55 kDa (Figure 3.2).

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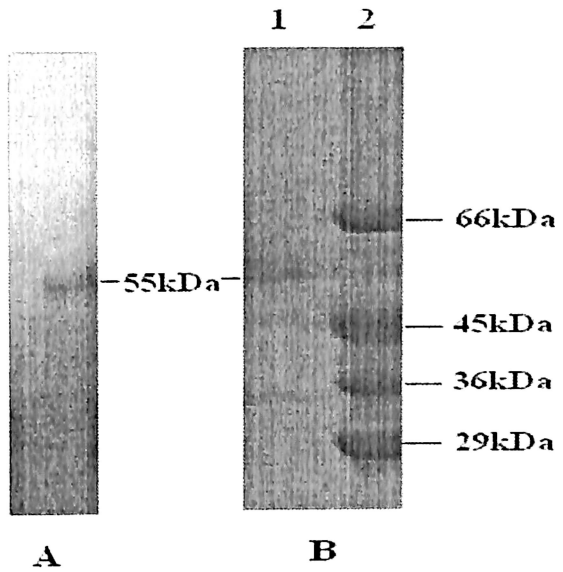


Figure 3.2: SDS-PAGE of Pseudomonas lipase

(A) DEAE sepharose purified sample. (B) Lane 1: Crude lipase. Lane 2 : Molecular weight markers

3.3.5 Optimum temperature and pH for production of lipase

Lipase activity has been plotted as a function of temperature (Figure 3.3A) and pH (Figure 3.3B). The optimum temperature of the isolated lipase was 30°C, whereas, its optimum pH value was found to be 7.

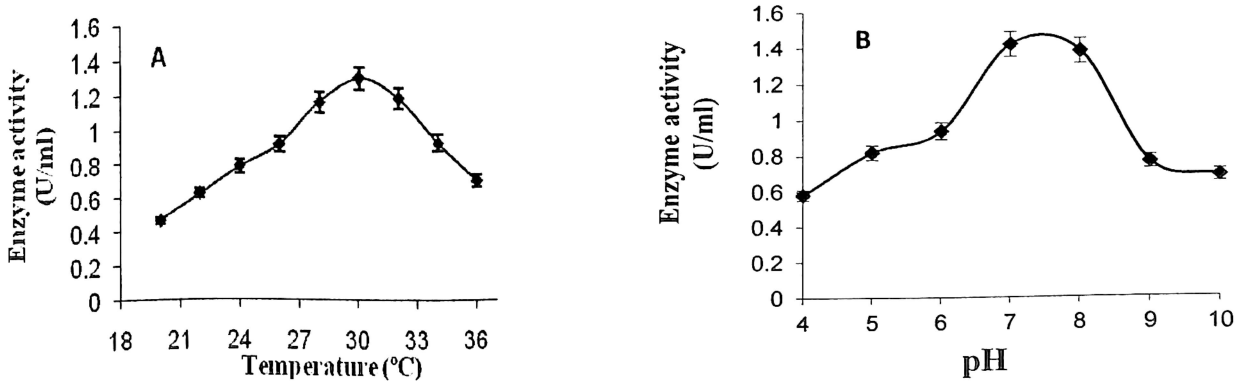


Figure 3.3 Temperature optima and pH optima.
A: Temperature optima and B: pH optima
Each datum point represents the average of the analysis of triplicate values represented by error bars.

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3.3.6 Thermal stability and storage stability of the isolated lipase

The activity of the isolated purified lipase powder retained ~98% of the initial catalytic activity when stored at 4°C and pH 7 for one month. A $t_{½}$ of nearly 23 months was calculated. The purified powdered lipase sample showed better stability compared to the lipase present in the cultured supernatant. The stability of the powdered lipase diminished steadily with the increasing temperature (Figure 3.4) from 62 days (at 20°C) to 87 minutes (at 80°C).

The enzyme has shown its activity (Figure 3.5) in a broad range of pH 4 to 10. The activity was increased initially with the increasing pH then decreased in the similar fashion. The activity of was drastically lost outside pH range of 4 to 10 and when boiled. The stability ($t_{½}$) of lipase activity at pH 7 was nearly 42 months and was found to be exceptionally stable at neutral to slightly alkaline pH in the range of 7 to 8.

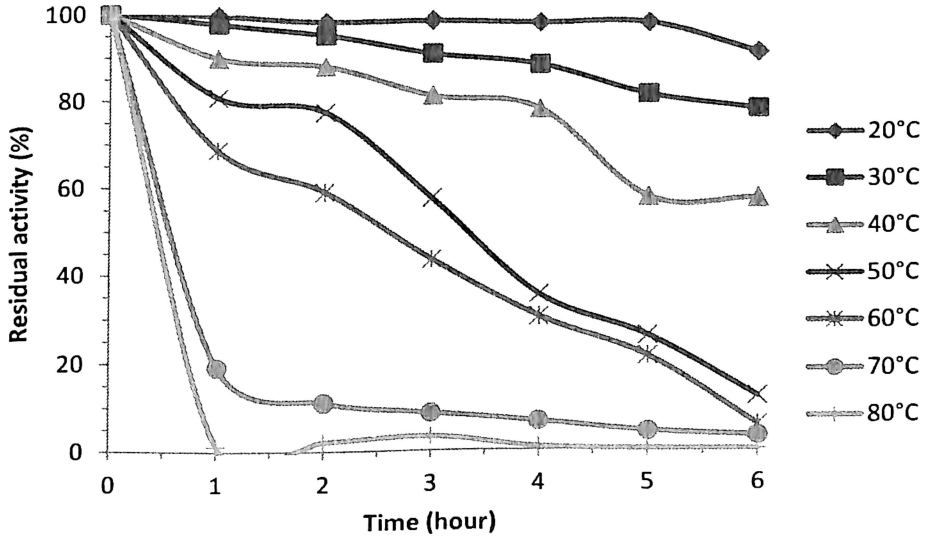


Figure 3.4 Temperature stability study of the isolated and purified lipase

[Each datum point represents the average of the analysis of triplicate values, with 100% activity corresponds to 21.0±0.03U/mg]

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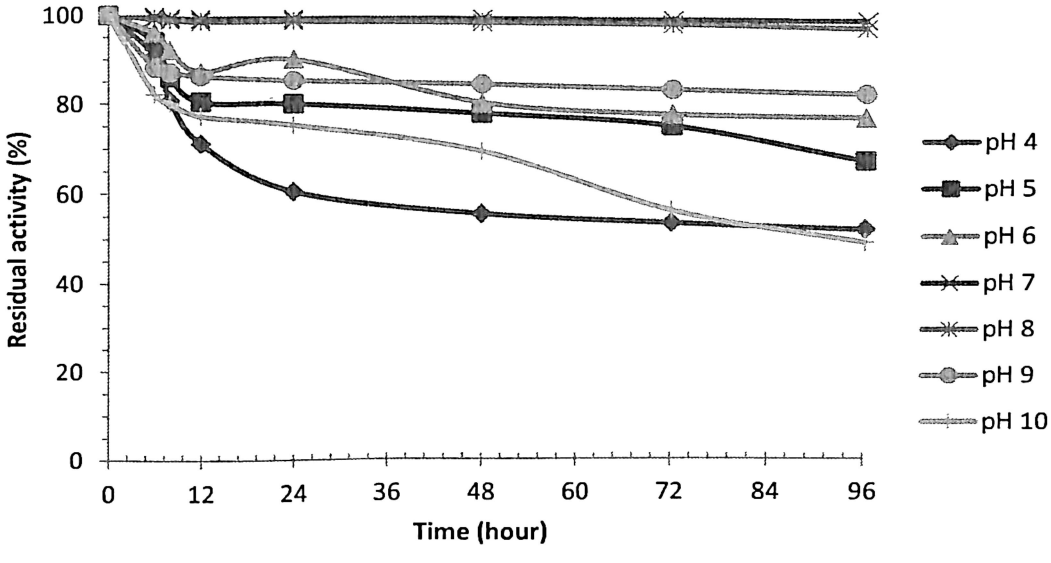


Figure 3.5 pH stability study of the isolated and purified lipase

[Each datum point represents the average of the analysis of triplicate values, with 100% activity corresponds to 21.0±0.03U/mg]

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3.4 Conclusions

A bacterial strain, identified as *Pseudomonas aeruginosa* PG01, produces highest lipase activity in the culture supernatant when sunflower oil was used as the sole carbon substrate. A significant production of lipase in burnt oil was also observed, which was about 75% of the activity obtained by using sunflower oil as the substrate. The extracellular lipase was successfully fractionated from the culture supernatant by ammonium-sulfate fractionation method, and then purified by DEAE-sepharose anion exchange chromatography. The specific activity of the ion-exchange column filtered purified lipase was increased to 2.59 fold. The molecular weight of the DEAE sepharose filtered purified *Pseudomonas* lipase estimated by SDS-PAGE was found to be 55 kDa. The optimum temperature and pH of the isolated lipase was 30°C and 7, respectively. The $t_{½}$ of the lipase at 4°C and pH 7 was nearly 23 months. The stability of the powdered lipase diminished steadily with the increasing temperature from 20°C to 80°C with $t_{½}$ of 62 days and 87 minutes at 20°C and 80°C, respectively.

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CHAPTER

Hydrolysis of vegetable oil by immobilized lipase

4.1 Overview

The biocatalytic production of biodiesel has been mostly studied by transesterification of vegetable oil using lipase biocatalyst in a single step reaction. However, the low yield of the product in transesterification reaction has been identified as one of the major constrain for developing a commercially viable biodiesel production process. An alternative biocatalytic approach that has not been seriously pursued is the two step reaction process, the first step being the hydrolysis of the vegetable oil, while the second step involves the esterification of the fatty acids liberated from the first step reaction for the production of biodiesel. Hydrolysis of fats and oils is also a well-known important activity in oleo chemical industries for the production of fatty acid, which is a renewable feedstock for industrial-scale processing to give value-added products for food, beverages, cosmetics, and medicinal applications (Hasan et al., 2006; Gandhi, 1997). In the fatty acid industry, hydrolysis of fats and oils is usually accomplished by a high temperature steam treatment method that operates with super-heated steam and high pressure (Brady et al., 1988). This high-temperature process is energy intensive and causes extensive degradation of the produced fatty acids. There is a growing interest to replace this chemical method with enzymatic methods as the later methods are normally operated under mild conditions in an energy efficient manner. The lipase is a lipolytic enzyme that functions at oil–water interface and generates less

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undesirable by products during hydrolysis of fats, and hence, its application potential as catalysts for hydrolysis of oil and fat has been increasingly focused (Hermansyah et al., 2007; Linfield et al., 1984; Park. et al., 1988; Moussata and Akoh, 1998; Zhao et al., 2007). Since interfacial activation is essential for catalytic activity of lipase (Zaks and Klibanov, 1985) various surface-active agents are employed to emulsify the fat in aqueous phase for enhancing the hydrolytic activity of lipases. However, majority of the surface-active agents utilized for the hydrolysis are synthetic and non-biodegradable in nature, thus, the effluent generated from the down-stream processing may cause environmental pollution if preventive measure is not taken. Biosurfactants are usually biodegradable surface-active agents. One of the main focuses of our work presented here is to utilize biosurfactant for the lipase-catalyzed hydrolysis of vegetable oil. Biosurfactants are surface active compounds produced by various living organisms such as, bacteria (Bryant, 1989), fungi (Kiran et al., 2009; Mukherjee et al., 2006) and other living organisms. Chemically biosurfactants are mostly of lipoprotein, glycolipid, and protein-lipid-sugar complex. *Pseudomonas strains* produce biosurfactant mostly of glycolipid in nature (Reiling et al., 1986). Application of immobilized enzyme for industrial process is a well-known fact that contributes to the process economy through reuse of the enzyme. Potential methods and support materials for immobilization of lipases have been reviewed (Cesar et al., 2007). Various organic and inorganic support materials such as Celite-545 (Shah et al., 2004), silica (Kumari et al., 2009), silica gel (Du et al., 2005), cellulose fabric (Luo et al., 2006), macroporous weak anionic resin beads (Shieh et al., 2003), magnetic chitosan microspheres (Xie and Wang, 2012), Sepharose-Q (Acosta et al., 2011) etc. have been reported for immobilization of lipase. Further on this line we are describing

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here a potential biodegradable support system for the immobilization of lipase to be used for the hydrolysis of a vegetable oil.

4.2 Experimental approach

4.2.1 Materials

Refined sunflower oil (*Helianthus annuus*) [containing saturates 12 %, and polyunsaturates 60 %; density 0.91 g/cc at 30°C] (SUNDROP, India) purchased from local market. Woven Muga Silk fiber (Silk-mat) produced by *Antherea assamensis* purchased from Sualkuchi (Silk village in Assam); Glutaraldehyde and Amberlite were purchased from Merk; Commercial powder lipase (E.C. 3.1.1.3) (Triacylglycerin lipase) from *Asperrgillius niger* was obtained from HIMEDIA. All other chemicals were of reagent grade and purchased from M/S AB Chemical. Polyurethane foams were collected from local market.

4.2.2 Cultivation of microorganism

The *Pseudomonas aeruginosa* PG01 was maintained in Pseudomonas-agar slant at 30°C. The organism was cultivated using 2% *n*-octadecane (as sole source of carbon for energy and growth) in a mineral media in 250 ml Erlenmeyer flask with partial modification as reported by Goswami and Singh (1991). Modified mineral media composition (in 1 litre distilled water) is as follows: $NaNO_3$----2g, $(NH_4)_2SO_4$----2g, Na_2HPO_4----3.61g; KH_2PO_4----1.75g; $MgSO_4 \cdot 7H_2O$----0.2g; $CaCl_2 \cdot 2H_2O$----0.05g; $FeSO_4 \cdot 7H_2O$----1mg; $CuSO_4 \cdot 5H_2O$----50µg; MoO_3----10µg; $MnSO_4$----10µg; pH was adjusted at 6.8 by using 1N HCl. The

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medium was sterilized by autoclaving at 121°C for 20 min. The flasks were agitated at 200 rpm at 32°C for growth of the organism.

4.2.3 Isolation and purification of biosurfactant

The biosurfactant was isolated and purified from the culture broth of an *n*-hexadecane grown *Pseudomonas aeruginosa* strain PG01 following the similar procedure described by Finnerty et al. (1984). The media composition and growth condition for harvesting the biosurfactant were similar to the methodology described in Chapter 3. The growth at late exponential phase (36 hours) was withdrawn and subjected to 1000×g at 4°C for 10 minutes to collect the cell-free supernatant. The biosurfactant was precipitated by adding concentrated hydrochloric acid to a final pH 3.5. The solution containing precipitates transferred to a separating funnel and mixed with equal volume of diethyl ether. Aqueous phase was discarded and organic phase was collected for biosurfactant isolation. Diethyl ether was evaporated from the extract in vacuum rotary evaporator and the biosurfactant was collected as brown mass. The collected brown mass was kept in a vacuum desiccators containing calcium oxide powder to remove traces of water following the procedure being described in the next section. The crude biosurfactant isolated by following the above procedure was purified by silica gel column. Solvents passed through the column were *n*-hexane, ethyl acetate and methanol in order of increasing polarity. Initially pure hexane, then mixture of hexane and ethyl acetate in gradually increased proportion of ethyl acetate, and then mixture of three solvents (*n*-hexane, ethyl acetate and methanol) with gradually increased amount of methanol was passed through the column. Finally pure methanol was passed through the column. The fractions were continuously monitored in thin-layer chromatography (TLC)

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plate using solvent systems of varying polarity. Analysis showed a major component and several minor components in the biosurfactant preparation. The major fraction, eluted by hexane, ethyl acetate and methanol at a ratio of 3:7:10 through the silicic acid column was collected. The solvent was removed by vacuum rotary evaporator and the biosurfactant was collected as dry brown viscous material.

4.2.4 Characterization of the biosurfactant

The chemical characteristic of the purified biosurfactant was established by FT-IR (Spectrum One, Parkin Elmer) and Q-ToF Premier Mass Spectrometer (M/S Waters) [Liquid Chromatography Mass Spectrometer (LC/MS MS), Waters Q-ToF Premier & Aquity UPLC]. The samples were obtained in negative ionization mode for Electrospray Ionization Mass-spectrometry (EIS-MS) study. The samples were prepared in 1:1(v/v) HPLC grade methanol/water with 0.01 % NaOH in 10 pg/µl concentration and filtered through 0.02µ filter paper and sonicated in ultrasonic bath for 20 minutes. The sample was then injected (2-5 µl) into the source at a rate of 10 µl/min using a carrier solvent of methanol to water ratio of 1:1(v/v) with 0.025% sodium hydroxide. The sampling cone voltage was set at 40 V. ESI-MS was operated at spray voltage 3.5 kV, and the heated capillary temperature was maintained at 120°C. Nitrogen was used as nebulizing and drying gas with flow rate of 2.5 and 8 l/min, respectively. Data were acquired over the mass range of m/z 200 to 1000. Ten to twenty scans were average-smoothed, baseline-subtracted and transformed using the Micromass transform software or maximum entropy program. The characteristics were identified from the corresponding *m/z* peaks using Masslynx 4.1 software provided with the

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instrument. This software includes both elemental composition and molecular mass calculator to determine the exact structure.

4.2.5 Rhamnose sugar estimation

Rhamnose sugar estimation in biosurfactant preparation was performed by orcinol–sulfuric acid methods. Orcinol reagent [0.2% (w/v) orcinol in 70% (v/v) H_2SO_4 in water] was prepared and kept cool. Standard samples (glucose) were prepared from 1 mg/ml stock solution with different concentrations: 0.5, 1.0, 1.5, and 2µg/ml. Reaction was started by adding 1.5 ml orcinol reagent to 0.5 ml of diluted samples in test tubes and kept in boiling water bath for 20 minutes. After cooling the samples in test tubes, the optical density was recorded at 450 nm against orcinol reagent–water blank.

4.2.6 Surface activity measurement

Surface tension and critical micelles dilution (CMD) were determined using a DuNouy Tensiometer (CSC No. 70335; CSC, Fairfax, VA, USA). All measurements were made on cell-free supernatent obtained by centrifuging the culture broth at 12,400 ×g for 25 minutes. For CMD measurements, the cell-free broth was diluted to different extent and the resulting surface tension values were recorded. The dilution at which the surface tension started to increase was considered as CMD.

4.2.7 Immobilization of lipase

Different immobilization approaches were studied for the immobilization of lipase isolated from *Pseudomonas aeruginosa* PG01 and commercial lipase. *P. aeruginosa* PG01 lipase of our own isolate was immobilized by physical absorption in hydrophobic

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polyurethane foam. In this case, polyurethane foam were cut into cubical shape (of size: 8mm × 8mm × 8mm) and the cubes of the foam were suspended in the cell free culture supernatant and then lyophilized until the supernatant dried completely. The stability of the immobilized support material was studied by suspending it in methanol, ethanol, hexane, acetone, and dichloromethane, respectively for 24 hours and then the physical deformation or weight loss, was examined. For assay of lipase absorbed in the polyurethane foam, the lipase immobilized cubes were suspended in a known volume of water for one hour under shaking condition at 30°C. The cubes were then separated and lipase desorbed into the aqueous phase was assayed following the standard method stated elsewhere. Also, the lipase was chemically immobilized on silk-mat using bifunctional cross-linking reagent glutaraldehyde. 0.1 g of commercial lipase was dissolved in 100 ml of 50 mM potassium phosphate buffer of pH 6.5. Silk mats were cut into 20 mm × 20 mm pieces (dry weight 0.038 g/piece) and washed successively with 0.04 N NaOH solution and 0.1 M sodium phosphate buffer of pH 7.0. The cleaned and washed silk mats were then suspended in the above lipase solution. The suspended solution was then mixed with suitable concentration of glutaraldehyde and kept at room temperature (25°C) for minimum 24 hours. The activity of the cross-linked enzyme on the silk mat was assayed after washing the fiber pieces with 50 mM sodium phosphate buffer of pH 8.0 for several times. The lipase immobilized silk mats were stored at 4°C for further use.

4.2.8 Assay of immobilized lipase

Enzyme activity in silk-mat immobilized lipase was determined by glycerol tributyrin-polyvinyl alcohol emulsion method, which is a partial modification of the method described

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by Ibrahim et al. (1987). 1% (w/v) polyvinyl alcohol was dissolved in 50 ml water by heating at 50°C-60°C. 2% (v/v) Glycerol tributyrin was dissolved in the above solution and sonicated to form an emulsion. 4 ml of this emulsion solution was mixed with 6 ml solution of sodium phosphate buffer (0.1M) pH 7.0 and one piece of lipase immobilized silk-mat in a 250 ml of conical flask and incubated for 20 minutes in orbital shaker at 30°C at 200 rpm. The reaction was terminated by adding 20ml solution of acetone to ethanol in the ratio 1:1(v/v). Free fatty acid liberated was titrated against 0.02 N sodium hydroxide solution using phenolphthalein as indicator. One unit of lipase activity is defined as the amount of enzyme that liberates 1 μmol of fatty acid per minute at 30°C.

4.2.9 Lipase catalyzed hydrolysis of vegetable oil

The reaction mixture consists of 8 ml of 0.1 M phosphate buffer of pH 7.2, 1 ml of sunflower oil, 1 ml of free lipase (10 mg/ml corresponding to 160 U/ml as per the unit of the powdered lipase mentioned by the supplier) solution or an equivalent quantity (in terms of protein) of the immobilized lipase, and Triton X100 or biosurfactant at a concentration of 20% higher than their respective Critical Micelle Concentration (CMC). The hydrolysis was carried out at 30°C, 150 rpm for 6 hours. The reaction was stopped by removing the immobilized lipase or with 20 ml of acetone-ethanol mixture (1:1 v/v ratio) for powdered lipase. Amount of free fatty acid liberated on the hydrolysis was monitored by titrating the supernatant against 0.04 N NaOH solution using phenolphthalein as indicator. After each cycle of operation the silk-mat was thoroughly washed with alkaline (pH 8.0) sodium phosphate buffer to use the immobilized lipase for the next cycle of the reaction.

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4.2.10 Statistical analysis

Results obtained were the mean of three or more determinations. Analysis of variance (ANOVA) was carried out on all data at p-value < 0:05.

4.3 Results and Discussion

4.3.1 Characterization of immobilized lipase

Uniform cubes (8 cm × 8 cm × 8 cm) of polyurethane foam were suspended in the lipase solution of activity 57 U/ml and lyophilized. The lipase activity of nearly 14.6 U/cube of the foam was detected. The morphological characteristics of the polyurethane foam are shown in Figure 4.1. The lipase immobilized cubes appeared yellow (Figure 4.1 B) probably due to the absorption of pigments produced by the *P. aeruginosa* strain in the culture supernatant during growth. Although the polyurethane foam immobilized lipase was stable over 3 months without loss of much activity, our effort of using this immobilized lipase for hydrolysis of vegetable oil was not successful due to lack of its operational stability. Polyurethane foam has been used by many researchers to immobilize other different enzymes, such as, beta-D-galactosidase (Hu, 1993), carbonic anhydrase (Kanbar and Ozdemir, 2010) etc. The polyurethane foam as immobilizing support matrix for lipase has also been reported for other substrate such as, butyric acid (Suzana et al.,1991), methyl methacrylate (Joel et al., 2003) etc. It was observed that the lipase immobilized on polyurethane cubes was slowly leached out during the continuous reaction for 24 hours at 32°C and no significant lipase activity in the immobilized matrix could be carried forward to

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the next cycle of the reaction. The hydrolytic activity of the immobilized lipase was also found to be low, since only 26.6% of the total vegetable oil used in the reaction mixture was hydrolyzed.

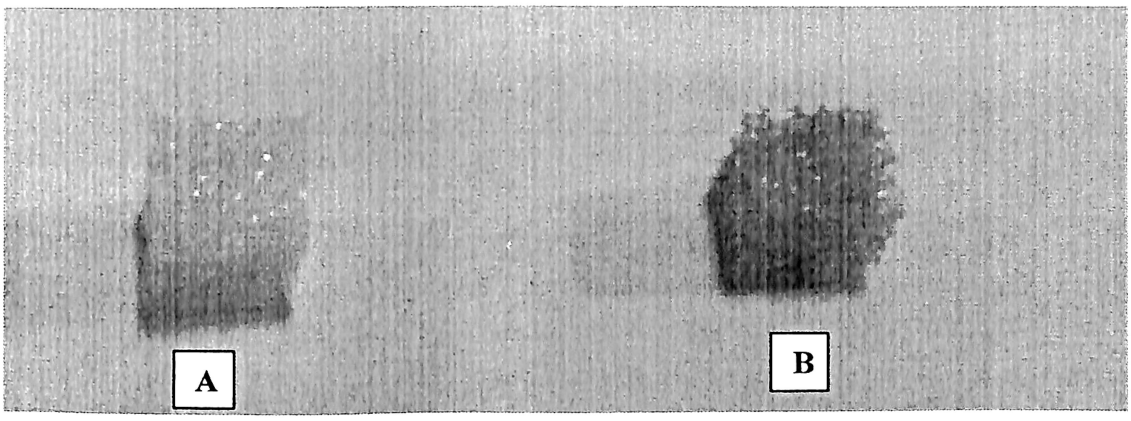


Figure 4.1: Physical absorption of lipase in hydrophobic polyurethane foam.
A: before immobilization of lipase and B: after immobilization of lipase.

As a second effort, we selected silk-mat as immobilizing support for the lipase considering the fact and assumption that silk fiber is a biocompatible and fairly hydrophobic matrix (Yang and Bochu, 2009; Sen and Babu, 2004), which may support better adsorption of enzyme like, lipase, bearing high hydrophobic surface area (Pawel et al., 1994), through hydrophobic-hydrophobic interactions. Moreover, in case the physical approach fails to stabilize the lipase immobilized on the silk-fibre, the lipase may be chemically immobilized on it due to the protein nature of the fiber that contains ample amino-groups as chemical linkers. Initially the lipase was physically adsorbed on silk mat and then covered with chitosan as binder. The morphological characteristics of the immobilized lipase on the silk-mat are shown in Figure 4.2. After immobilization, some white patches appeared on the fibers of silk-mat which were not there before immobilization, as evident from the morphological features.

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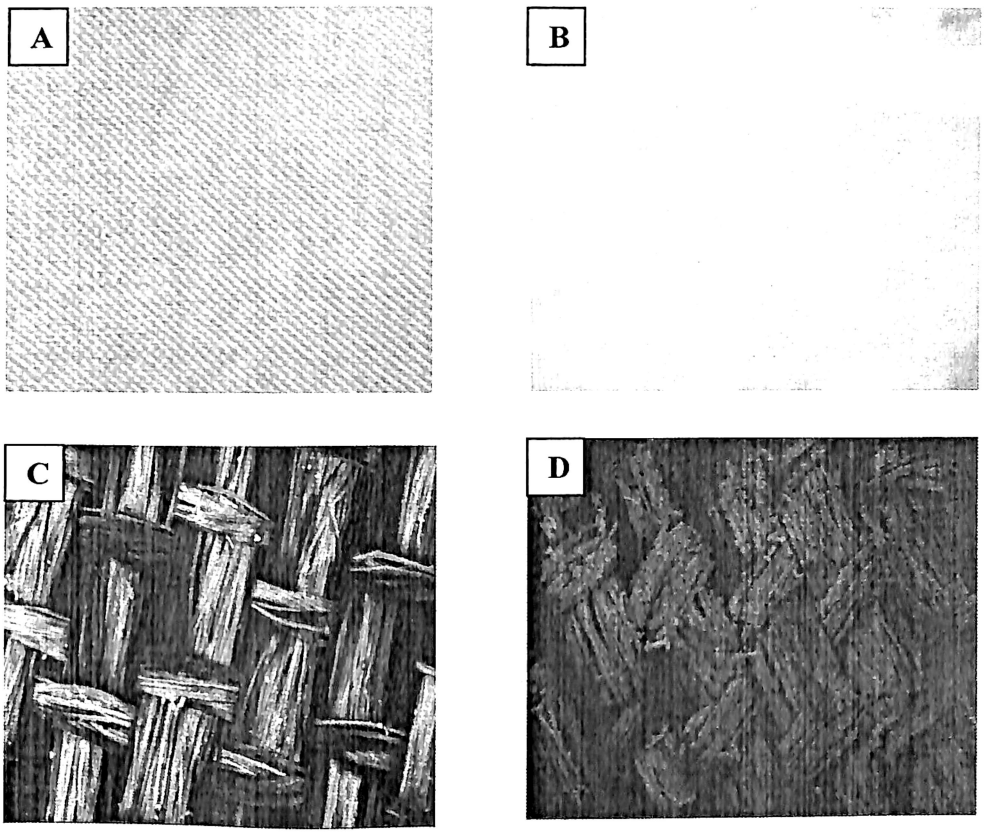
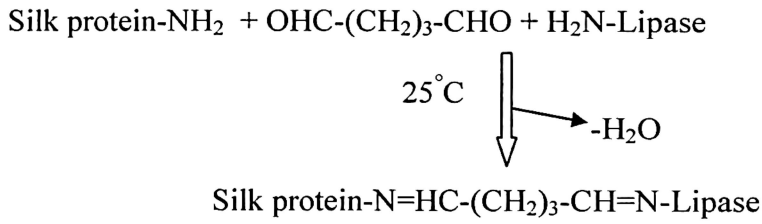


Figure 4.2: Morphological characteristics of the lipase immobilized silk-mat.
A: before, and B: after immobilization. The images were captured by a camera (make: Nikon Corp., Japan). C: before, and D: after immobilization. The images were captured by scanning electron microscope (make: LEO, model no. 1430vp) at a magnification of 70×

However, similar to the result as obtained by using the polyurethane foam, the operational stability of the immobilized lipase was found to be very poor. It was observed that upon washing with alkaline phosphate buffer (50mM, pH8) the chitosan bound lipase on silk-mat was considerably leached out, and the residual lipase activity in the washed silk mat was drastically reduced. To get rid of the leaching problem, the lipase was immobilized on the silk mat through chemical cross-linking using glutaraldehyde as the cross-linking agent following the reaction scheme 4.1.

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Scheme 4.1: Chemical immobilization of lipase on silk-mat

The loading of the lipase on the mat was optimized at room temperature (25°C) by varying the parameters namely, concentration of glutaraldehyde, and lipase treatment time, while keeping the other parameter constant. The concentration of glutaraldehyde was found to be critical on the loading of the enzyme on silk-mat. The optimum concentration of glutaraldehyde was found to be 5 % (v/v) as shown in Figure 4.3. The activity of the enzyme in the fiber declined beyond this concentration of glutaraldehyde. On prolonged incubation of the reaction mixture beyond 24 hours, with the said concentration of glutaraldehyde, the enzyme loading was only marginally increased and reached the maximum loading of 59.16 U/g-silk-mat at 27 hours of incubation.

The morphological change of the silk fibers upon immobilization of the lipase was also detected by scanning electron microscopy. For clarity of the morphological differences before and after immobilization, the resolution was captured for single silk-fiber. Under Scanning Electron Microscopy some white patches in the fiber structure were observed after the lipase treatment (Figure 4.4B) of the silk mat, which was not visible before lipase treatment (Figure 4.4A), which postulate the binding and loading of the protein.

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4.3.2 Stability and loading density of lipase chemically immobilized on silk-mat

The storage stability of the immobilized lipase was found to be very high as the activity of the lipase was almost retained for more than nine months at 4°C. Negligible loss of the hydrolytic activity of the silk-mat immobilized lipase was noticed on storage. However, at room temperature the immobilized lipase activity decreased per week was 2.83 U/g-silk-mat from the initial loading of 59 U/g. The activity of the equivalent powdered lipase decreased per week was 2.91 U.

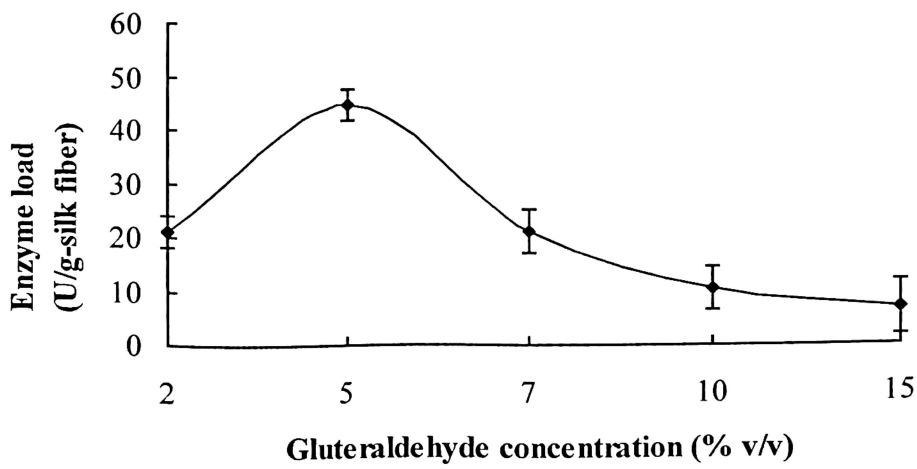
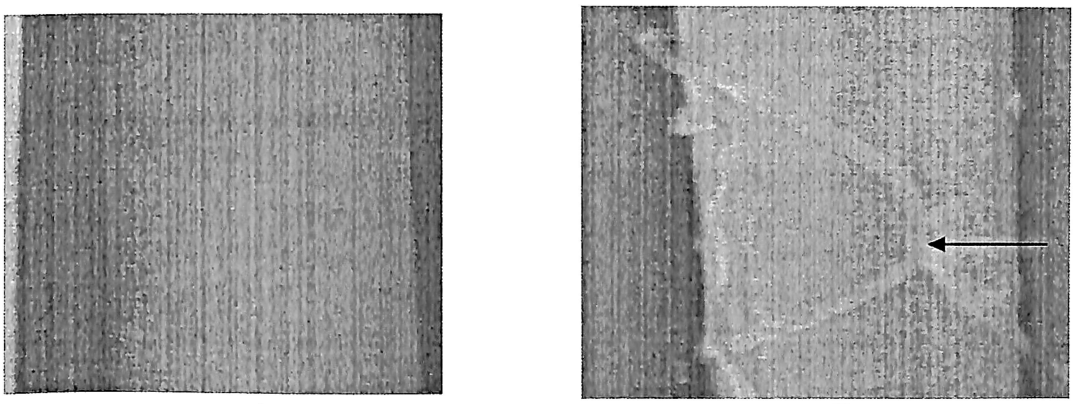


Figure 4.3: Optimization on the concentration of glutaraldehyde for enzyme loading on the silk-mat at 24hours of incubation

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A B

Figure 4.4: Scanning Electron Microscopic image (magnification 1.75KX) of silk-mat before and after immobilization of lipase (SEM make: LEO, model no. 1430vp)

Although various immobilization techniques have been developed, chemical immobilization of enzymes on a suitable support could possibly furnish long term mechanical stability and chemical durability provided the chemical link between the enzyme and the support matrix does not interfere with the functional activity of the enzymes. Further on this line we describe here the silk-mat as potential biodegradable immobilizing support system for the lipase to be used for the hydrolysis of a vegetable oil. This effectively resolves major technical obstacles in term of enzyme stability and tolerance against methanol as co substrate. Silk-mat as biocompatible immobilizing support matrix for other enzyme like cholesterol oxidase has been reported (Saxena and Goswami, 2010). However, the utilization of this biomaterial for immobilization of lipase is not known.

4.3.3 Isolation and analysis of biosurfactant

The production of biosurfactant in the culture broth of the bacterial strain was evident from the formation of massive foam as shown in the Figure 4.5. Biosurfactant was

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successfully isolated from the culture broth of (*Pseudomonas aeruginosa* PG01) following the method described earlier (section 4.1.2).

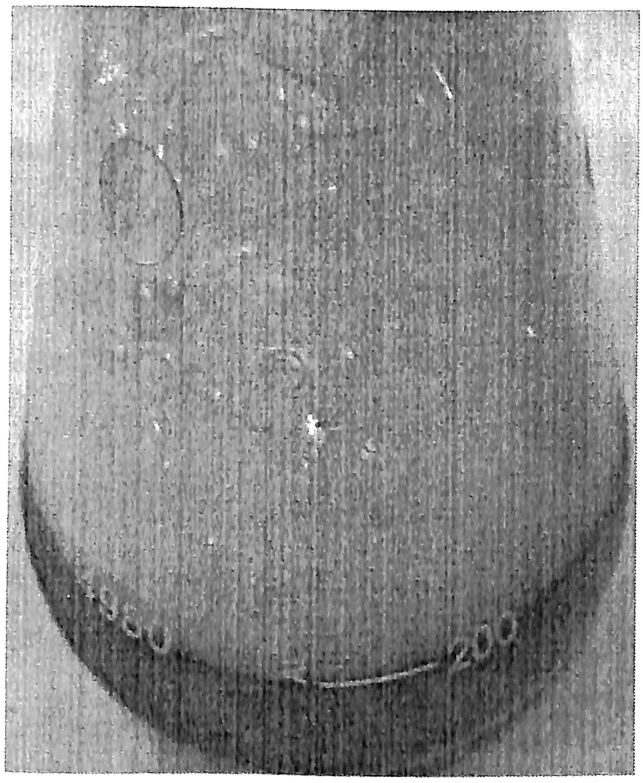


Figure 4.5: Physical appearance of the culture broth containing biosurfactant in the shake flask of *n*-hexadecane grown *Pseudomonas aeruginosa* strain PG 01.

FT-IR analysis (Figure 4.6) of the purified biosurfactant in KBr showed strong broad absorption at 3,419 cm^{-1} indicating the presence of –OH group in the molecule, while, absorption at 1,651 cm^{-1} indicates the stretching mode for >CO–O bond. The bands at 2,925 cm^{-1}, 2,854 cm^{-1}, 1,470 cm^{-1}, and at 1,378 cm^{-1} reflect aliphatic chains ($-CH_3$, $-CH_2-$) of the fraction. The absorption at 1,740 cm^{-1} was due to lactone carbonyl absorption, indicating that the product contains aliphatic hydrocarbon as well as sugar-like moiety. No characteristic bands corresponding to the –N-H stretching and deformation mode was detected in the spectra. Thus, the presence of peptide or amino moiety in the biosurfactant

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molecule is ruled out. Chemical analysis for sugar showed the presence of rhamnose sugar in the purified biosurfactant. Following the methodology described earlier in section 4.2.5 (Orcinol method for rhamnose sugar estimation) an amount of 0.1317 microgram of rhamnose sugar per gm of the isolated purified brown sample was detected. ESI-MS analysis (Figure 4.7) showed molecular ion peak (m/z) at 701.98. This m/z value is found to be similar for the rhamnolipid structure Rha-Rha-C12:1-C12 and Rha-Rha-C10-C14:1 reported by Haba et al. (2003). The above findings suggest the rhamnolipid nature of the biosurfactant isolated from the n-hexadecane grown culture broth of *Pseudomonas aeruginosa* PG01.

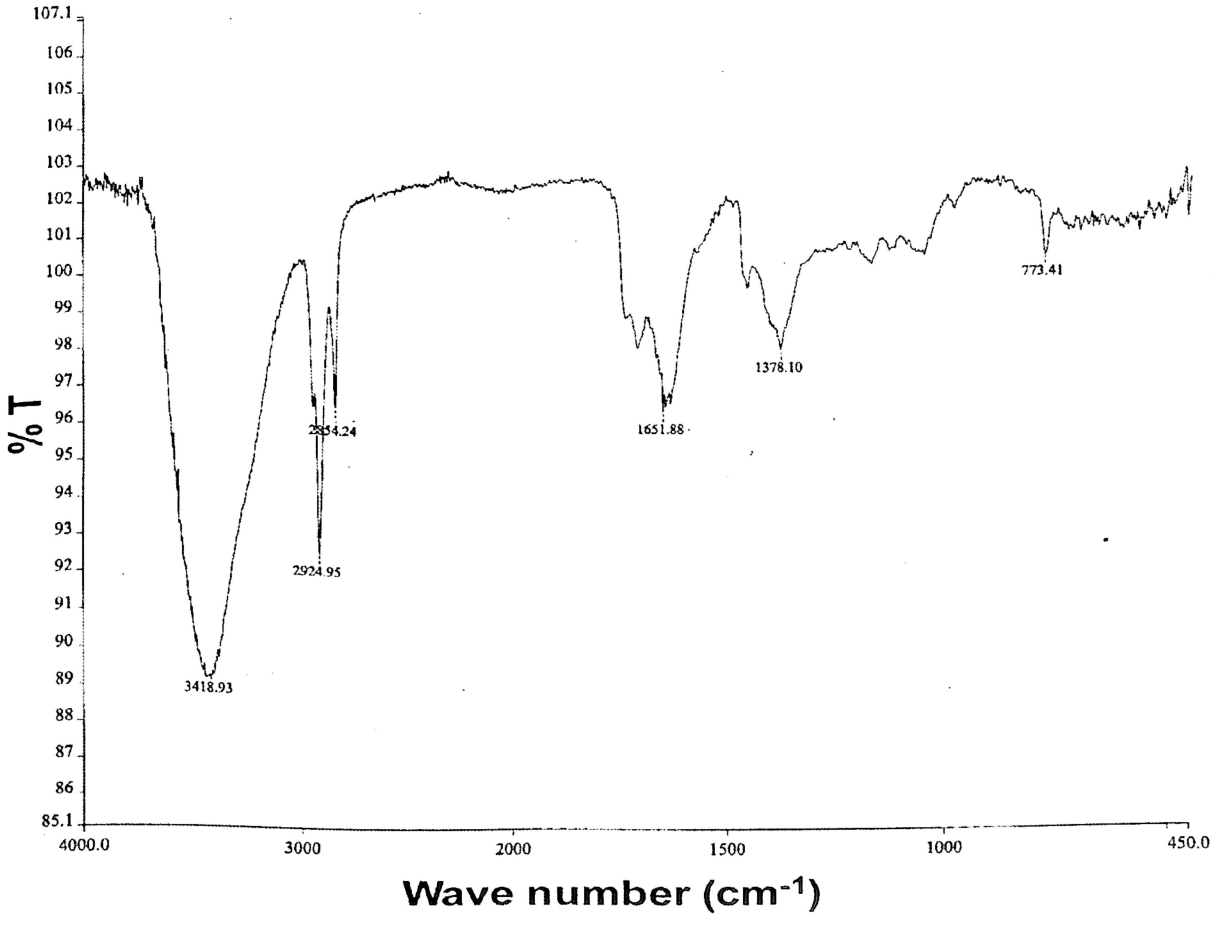


Figure 4.6: FT-IR spectra of the isolated biosurfactant

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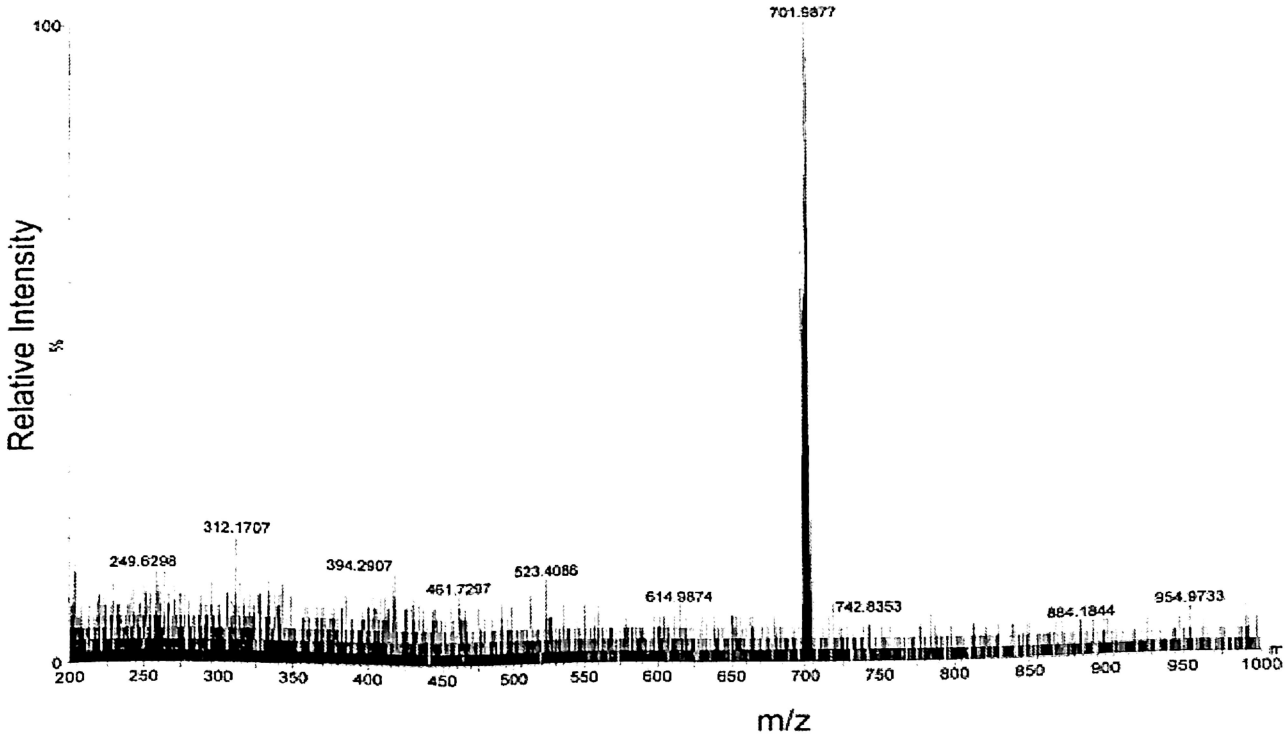


Figure 4.7: ESI-MS spectra of the pure biosurfactant

There are various microbes reported in the literatures capable of producing surfactants with a wide variety of different molecular structures, which have the ability to emulsify various hydrophobic substrates in aqueous medium. Most of these biosurfactant producing microorganisms produce such surface active agents on hydrophobic substrates such as *n*-alkanes, fatty acids or plant oils (Goswami and Singh, 1991; Lang, 2003; Cunha et al., 2004; Costa et al., 2006). Lin (1996) has reviewed the properties, physiological functions, biosynthesis, genetics, assays, production, recovery and application of biosurfactant. Many *Pseudomonas* strains are known to produce rhamnolipid biosurfactant (Amin and Radwan, 1997; Zhang and Miller, 1994; Xiulin et al., 2005; Ochsner et al., 1994; Beal and Betts, 2000). The functional properties and productions of rhamnolipid biosurfactant from *Pseudomonas* strain have been extensively studied (Haba et al., 2003; Reiling et al., 1986).

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4.3.4 Hydrolysis of Sunflower Oil with Lipase

Lipase catalyzed hydrolysis of sunflower oil in two phase system (oil/water) was studied using free or silk-mat immobilized lipase (Table 4.1). The hydrolytic activities of both the free and immobilized lipases were found to be poor even after changing the various operational parameters like, temperature, shaking speed, and substrate–catalyst ratio, thus appeared unsuitable for the process. The poor catalytic activity of the lipase was attributed to the low surface to volume ratio of the oil in the reaction mixture resulting in low substrate availability for the catalysis as well as lack of interfacial activation of lipase in this two-phase system. We proposed to promote this hydrolysis of oil in water emulsion system. A non-ionic surfactant, Triton X100, ultrasonic vibration, and the rhamnolipid biosurfactant were used to generate the emulsion, which were then used for lipase-catalyzed hydrolysis of sunflower oil. The concentration of the Triton X100 and rhamnolipid were used at a concentration of 20% higher than their respective critical micelle concentrations of 0.2×10^{-3} M and 0.0213×10^{-3} M, respectively, to ensure the sufficient emulsification of the oil used for the hydrolytic reaction. The stability of the emulsions created by these chemical surfactant, biosurfactant, and ultrasonication was studied at room temperature and found that the stability of the emulsions generated by the biosurfactant and Triton X100 were nearly equivalent up to 24 h of incubation (Figure 4.8). However, when these emulsions were kept at 4°C for a week the stability of the Triton X100-mediated emulsion was largely depleted, whereas, the stability of the biosurfactant-mediated emulsion was retained even more than 50% of the original value.

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Table 4.1: Hydrolytic activity of the free and immobilized lipase under different conditions of the sunflower oil.

Condition of oil in the medium	Activity (U)		
	Free lipase	Immobilized lipase	Retention (%)
O/W with or without ultrasonication	Trace (<5)	Nil	Nil
O/W emulsion using Triton X100	48.01	36.11	75.20
O/W emulsion using biosurfactant	59.65	48.73	87.50

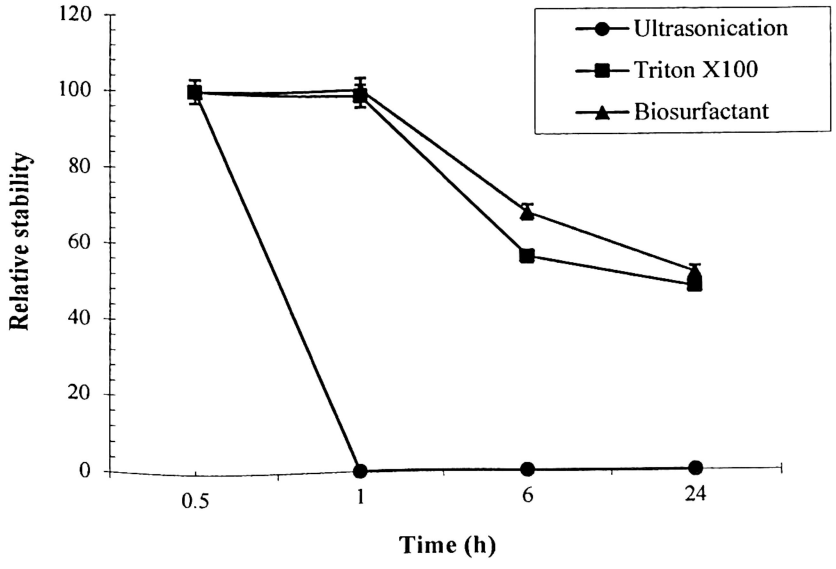


Figure 4.8: Stability of emulsions generated by utrasonication, biosurfactant, and Triton X100

4.3.5 Activity and stability of immobilized lipase

The activity of the immobilized lipase was studied for the hydrolysis of emulsified sunflower oil created by using biosurfactant and Triton X100 separately (Table 4.1). The

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hydrolytic activity of the lipase increased when the oil was emulsified in the reaction mixture, and this increase was higher in the biosurfactant-mediated emulsion of the oil than the Triton X100-mediated emulsion. The activity of the lipase was nearly void when ultrasonicated emulsion was used. The hydrolytic activity of the lipase for the oil emulsion created by the Triton X100 and biosurfactant were 36.11±0.96 U and 48.73±1.26 U, respectively. Thus, nearly 25.9 % higher activity of the immobilized lipase was demonstrated in biosurfactant-mediated emulsion over Triton X100-mediated emulsion. The reason for enhanced catalytic activity of the lipase for the hydrolysis of biosurfactant mediated emulsion is uncertain yet. However, it is suggested that this surfactant from the biological sources might promote better interfacial microenvironment for enhanced activity of the lipase. Further investigation on the interfacial microenvironment created by the biosurfactant is warranted to unveil the fact. The retention of activity of the immobilized lipase in Triton X100-mediated hydrolysis was nearly 12% less than the biosurfactant mediated hydrolysis as evident from the data shown in Table 4.1. This implies the partial inhibitory effect of Triton X100 as compared to the effect of corresponding biosurfactant on the immobilized lipase-mediated hydrolysis of the oil.

4.3.6 Reusability of Lipase

The reusability of the silk mat-immobilized lipase for the hydrolysis of sunflower oil was studied by repeating the hydrolytic reactions with a single set of immobilized lipase (Figure 4.9).

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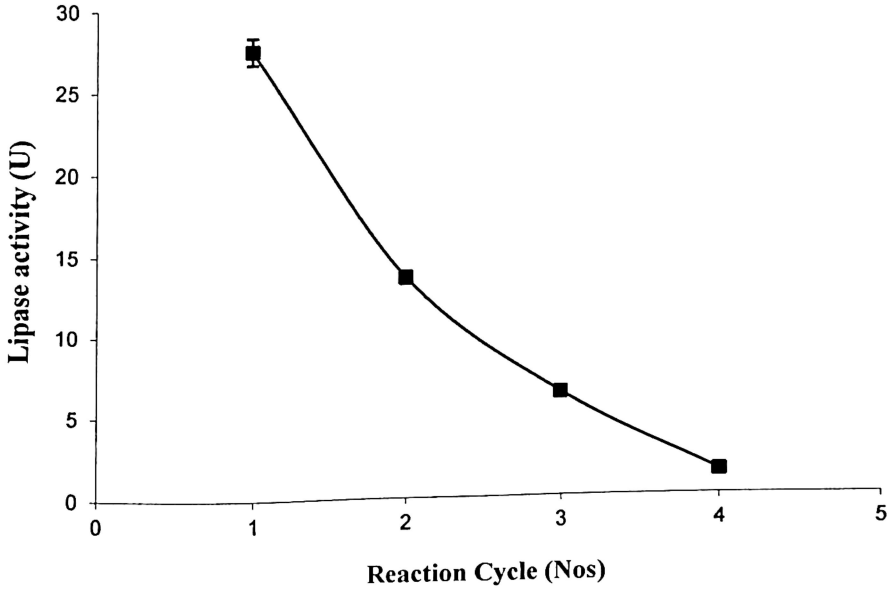


Figure 4.9: Reusability of the enzyme immobilized on silk-mat for the hydrolysis of vegetable oil.
(The error bars represent the standard error for triplicate measurements)

Each reaction was carried out at 30°C and 150 rpm for 6 hours. After each cycle, the mats (each mat of 20 mm × 20 mm sizes) were subjected to stringent washing with methanol and hexane in 1:1 ratio, followed by sodium phosphate buffer of pH 8.0. The yield of the fatty acids in the second, third, and fourth cycles were 49.45%, 22.91%, and 5.09%, respectively, of the yield obtained in the first cycle, considering the yield 27.5 mM in the first cycle as 100%. We observed that to regain sufficient activity after each cycle of the operation the immobilized lipase required thorough washing with slightly alkaline (pH 8.0) sodium phosphate buffer. The reason is ascribed to the dissolution of the product fatty acids associated with the lipase by the alkaline buffer solution and thereby making the active sites of the lipase free for further catalysis.

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4.4 Conclusions

The silk mat, which is a known biodegradable and renewable biomaterial, as support for the chemical immobilization of lipase, is reported. The chemically immobilized lipase was successfully used for the hydrolysis of sunflower oil for the production of fatty acids. This lipase catalyzed hydrolysis was found to occur effectively when the substrate sunflower oil was used as emulsion, mediated by either a chemical or a biological surface active agent, namely, Triton X100 and rhamnolipid, respectively. Further, we demonstrated that the rhamnolipid was a better surface-active agent than the Triton X100 for the hydrolysis as evident from the 25.9% higher lipase activity, realized by using emulsion created by biosurfactant over Triton X100. Although, the efficiency of the immobilized lipase was nearly 12% less than the free lipase on the biosurfactant-mediated hydrolysis of sunflower oil, the immobilized lipase could be reused even up to the third reaction cycle in this biosurfactant-mediated conversion, thus appearing as a potential industrial biocatalytic process for hydrolysis of vegetable oil.

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Indian Institute of Technology Guwahati

CHAPTER

Transesterification of vegetable oil using immobilized lipase for production of biodiesel

5.1 Overview

Lipase catalyzed transesterification has received renewed interest over the conventional transesterification processes because of room temperature reaction condition, reuse of catalysts, ability to esterify both glycerides and free fatty acids in single step, and production of glycerol stream with minimal water content and little or no inorganic material (Shah et al., 2004). However, the cost of the lipase is one of the major obstacles in developing lipase-based processes for commercial applications. Application of immobilized lipase in biocatalytic processes substantially averts the cost factor, thus contributes to the economic viability of the process for commercial applications (Shieh et al., 2003). The immobilization, however, should not affect the functional activity and stability of the lipase being used as catalyst for the transesterification reaction. The role of the immobilizing support material is therefore, critical for the success of the transesterification process. Application of various lipase immobilization support matrices for biodiesel production has been reported with mixed results (Yucel, 2011; Denise et al., 2011). Recently, much attention has been paid to utilize biocompatible materials for the immobilization of lipase to overcome the loss of lipase stability commonly occurred due to the immobilization (Chand et al., 1997; Wang et al., 2011; Huang et al., 2011). The other

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equally important factor that governs the functional activity and stability of the immobilized lipase is the kind of immobilization. Different approaches such as chemical cross-linking (Karimpil et al., 2011), encapsulation (Gao et al., 2010; Yang et al., 2009), adsorption (Moreno and Giraldo, 2011) etc., have been utilized for immobilization of lipase on various support systems for the transesterification reaction. The success of such approaches, however, could not be convincingly generalized so far and showed variation across enzymes of different sources (Deng et al., 2004; Leung et al., 2010; Antczak et al., 2009; Banerjee and Chakraborty, 2009; Bajaj et al., 2010; Tan et al., 2010). The additional important issue that needs to be considered for developing the biocatalytic process is an efficient bioreactor for facilitating the reaction to achieve high product yield. Different bioreactors for the lipase catalyzed transesterification of vegetable oil for the production of biodiesel has been reported such as biphasic bioreactor (Kee et al., 2010), membrane bioreactor (Deng et al., 2005), packed bed bioreactor (Huang et al., 2008), fluidized bed bioreactor (Sanchez et al., 2006), stirrer tank bioreactor (Mendes et al., 2009) etc. This chapter describes transesterification of vegetable oil in a small scale packed bed reactor using lipase immobilized on a novel material of biological origin.

5.2 Experimental approach

5.2.1 Materials

Powdered lipase (73000 U/g) from *Aspergillus niger* (RM-1265) was obtained from Himedia Laboratory Pvt. Ltd., India. Sodium hydroxide, methanol, glutaraldehyde, and para nitophenyl acetate (PNPA) were obtained from Merk, India. Sunflower oil was procured from local market. Silk cocoons produced by *Antheria assama* were purchased from Sualkuchi, the silk village of Assam, India.

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5.2.2 *Immobilization of lipase*

The lipase was immobilized in fibrous silk cocoon. For that the inner worms were removed first and then the outer shells were collected and boiled for 2 hours to remove any sort of dust and microbial contamination. The cleaned cocoons were dried under air at room temperature. Each dried cocoon was then cut vertically and horizontally into eight small nearly uniform pieces. The sliced pieces of cocoons (total 4.69 grams) were treated with alkali (0.8 N NaOH) for 2 hours to remove gums and Sericin (a protein) and then washed with methanol (250 ml) and double distilled water (500 ml). The cleaned sericin free cocoons were then treated with a mixture of equal volume of glutaraldehyde (5%) and lipase solution (1g powdered lipase in 0.1M sodium phosphate buffer of pH 7.2) for 24 hours at room temperature. The lipase immobilized cocoon pieces were then washed with the 50 mM sodium phosphate buffer followed by lyophilization for 8 hours to dryness and then kept at 4 $^{\circ}$C for further use.

5.2.3 Lipase activity assay

The activity of free and immobilized lipase was measured following a reported method using PNPA as a substrate (Salihu et al., 2011) with some minor modifications. The activity of the enzyme was determined by measuring the absorption of the product *p*-nitrophenol formed in the solution at 410 nm. The reaction mixture (1.0 ml) consists of 0.5 ml 100 mM sodium phosphate buffer, 2mM *p*-nitrophenyl acetate, 5% CH_3CN, and 0.25 ml of enzyme solution (powdered lipase in 0.1M sodium phosphate buffer) or suitable amount of silk cocoon immobilized-lipase in 0.25 ml 100 mM sodium phosphate buffer. A control was run simultaneously using the same sodium phosphate buffer. The reactions were initiated by adding

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0.15 ml of 50 mM *p*-nitrophenyl acetate (initially dissolved in dry CH_3CN) to the buffer solution containing the enzyme that was pre incubated at 37°C for 3 minutes. The mixture was then incubated for 2 minutes at 37°C. The reaction was stopped by adding 1 ml acetone and then clarified at 10000 ×g. The clear colored (pale yellow) solution was monitored for the formation of *p*-nitrophenol by measuring absorbance at 410 nm. One unit of the lipase activity was defined as the amount of enzyme which liberates 1µmol of p-nitrophenol per minute at 37°C. Experiments were carried out in triplicate and the data were considered with standard error within 5% at a confidence level of 0.85.

5.2.4 FESEM analysis

The morphology of the silk fiber on the cocoons surface before and after immobilization of lipase was studied by field emission scanning electron microscopy (FESEM) (instrument model: ZEISS, make: Sigma, Germany). The sample was imaged by scanning with high-energy beam electrons at 20 keV in a raster scan pattern. The sliced dry silk cocoons, which are non-conducting in nature, were initially coated with gold under vacuum. The dry specimens were then mounted on a specimen stub using an electrically-conductive double-sided adhesive tape.

5.2.5 Bioreactor for transesterification of sunflower oil

The bioreactor used for the transesterification reaction is shown in Figure 5.1. Lipase-immobilized cocoons were packed in the column under normal atmospheric pressure and room temperature (25°C). Total volume of the packed bed column was 37.17 ml, packed volume was 23.36 ml, and void volume was 13.81 ml. Nearly 10 ml space was left above the packed column,

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for retention and recirculation of the reaction mixture. A mixture of methanol and sunflower oil (250 ml in various ratio v/v) was sonicated in ultrasonic bath and taken in a three-necked round bottom glass container. Recirculation of the reaction mixture through the glass column containing silk-cocoon immobilized lipase was facilitated by means of a peristaltic pump (make: Miclins; model no.: PP20) at an optimized flow rate of 1.6ml per minute. Continuous mixing of the oil-alcohol was confirmed by magnetic stirrer. The temperature of the water jacket was maintained by connecting the inlet and out let terminals of the jacket to a digital temperature controlled water bath (make: Thermo Haake; Model no.: P5).

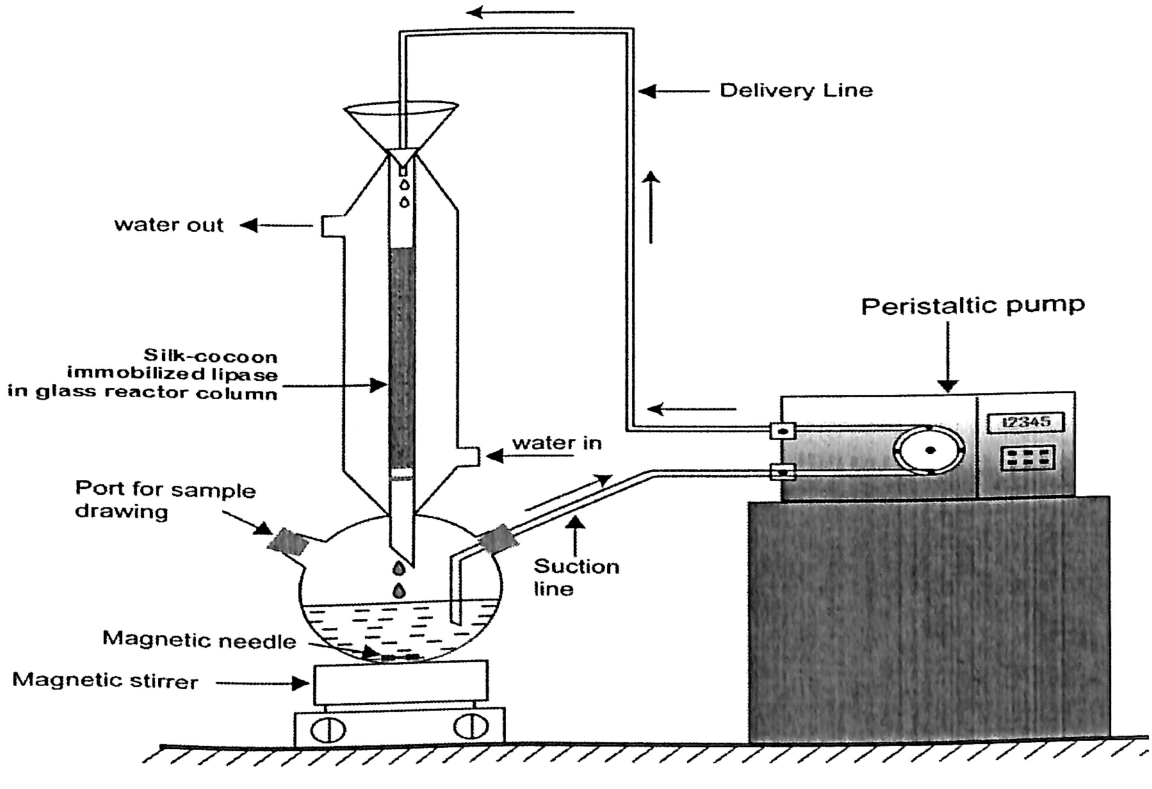


Figure 5.1: Bioreactor for silk-cocoon immobilized lipase catalyzed transesterification reaction for biodiesel production

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5.2.6 Product analysis

The transesterification of sunflower oil for the production of biodiesel was carried out in the bioreactor containing lipase immobilized cocoon matrix. The reaction was carried out at 25°C using reservoir substrate concentration of 25% (v/v). Samples, each of 1ml, were withdrawn at different time intervals for gas-chromatographic analysis. The product was analyzed by injecting the sample in thermo-capillary column (make: M/S Thermo Electron Corporation, model: TRACE™ TR-Wax MS) fitted on the gas chromatography equipment (make: Varian, model: CP-3800). The column has an internal diameter of 0.25mm, film thickness of 0.25μm, and length of 30m; with the phase type of polyethylene glycol. The parameters used in the analyses were: carrier gas, nitrogen (flow rate 2 ml/min); detector, FID; injector temperature, 28°C; and column-oven temperature, 230°C. For identification of the product in the chromatogram, the standard methyl esters (Sigma, USA) as well as the pure methyl esters of the fatty acids, produced chemically from sunflower oil using alkaline transesterification method, were used. Based on the total area of the major methyl esters in the chromatogram, the quantitative estimation and yield of the product were calculated. Although sunflower oil contains different fatty acids of varying chain lengths and different degree of unsaturation, yet it contains three major fatty acids (Ihara et al., 1991) namely, linoleic acid (C 18:2), oleic acid (C 18:1), and palmitic acid (C 16:0). During calculations, the total area under the three major ester peaks appeared in the chromatogram were considered. In order to compare the wholesomeness of the methyl ester formed during biocatalytic transesterification, pure methyl ester was initially prepared from sunflower oil by sodium hydroxide catalysis. For the synthesis of methyl esters of sunflower oil using chemical catalyst, 50 ml refined sunflower oil, 12.5ml methanol, and 0.4gram of anhydrous sodium hydroxide were mixed in a 250ml Erlenmeyer flask sealed (air tight) with

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cork and kept in an orbital shaker at 150 rpm and the 55°C for 8 hours. The reaction mixture was then taken in a separating funnel and the bottom layer of glycerol was separated out. Un-reacted methanol was separated by vacuum distillation at 65°C using vacuum rotary evaporator (model: Hahnvapour, make: M/S Hahnshin Scientific Co.). The remaining liquid was washed four times with double distilled water by hand shaking in a separating funnel. The pure methyl ester of sunflower oil so obtained was dried by anhydrous sodium sulphate and kept in a glass bottle with airtight cork at 4°C, for further use.

5.2.7 Statistical analysis

All the data presented in this study were the mean of three or more experiments with a variation within 10%. Analysis of variance was carried out for the statistical data, at p-value < 0.05 following a standard method (Kline and McClintock, 1953). When the experimental data value was found to be significant, critical difference was calculated by multiplying the standard error of difference with the corresponding error values at 5% level of probability. Significant differences among the means were determined by using least significant difference test.

5.3 Results and Discussion

5.3.1 Morphological study of lipase immobilized silk cocoons

The silk cocoons used for lipase immobilization were ovular in shape and brown in color as shown in Figure 5.2. The average length and diameter of the cocoons were 5 cm and 2 cm,

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respectively. The average weight of the cocoon was 293.67 mg. The lipase was successfully immobilized into silk cocoons matrix using glutaraldehyde cross-linking which is evident from the FESEM images (Figure 5.3). Figures 5.3 (A) and 5.3 (B) show the FESEM images of the silk-cocoon fiber before and after immobilization of the lipase respectively. Distribution of grey white patches were observed after immobilization which were absent in native cocoons. The average fiber diameter of the cocoons was also increased from 35.3 μm to 53.5 μm after the immobilization of lipase (Figure 5.3B) as revealed from the FESEM image [software: SmartSEM® (Zeiss)] at a magnification of 400 ×.



Figure 5.2: Silk cocoon from *Antheria assama*.

[Image was captured by a camera (Make: NIKON D 200) at F-50 mm.]

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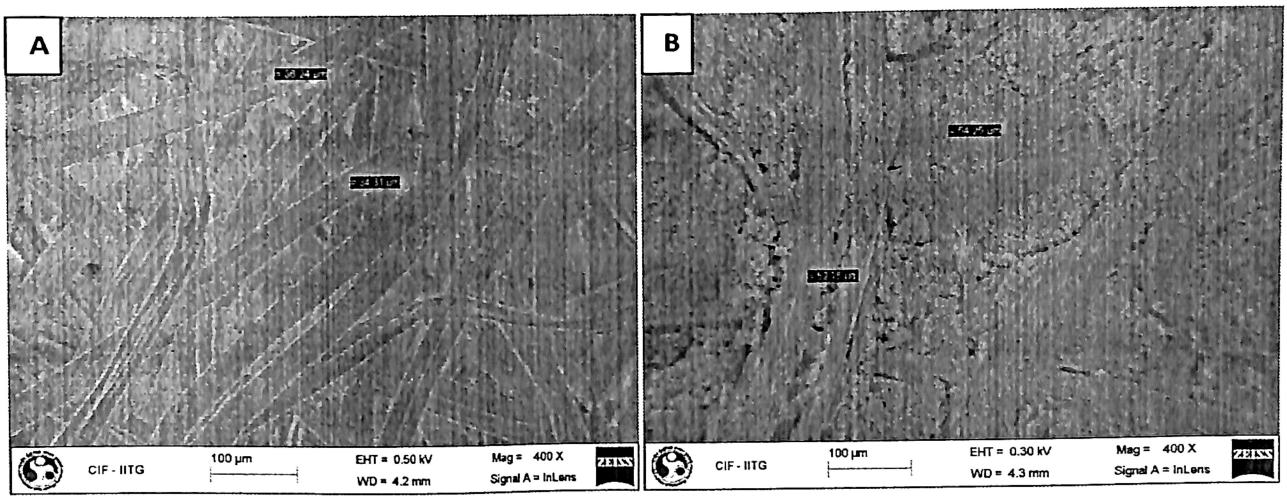


Figure 5.3: FESEM photograph (magnification: 400×) of silk fibre of silk cocoons: A: before lipase immobilization, and B: after lipase immobilization.

5.3.2 Lipase loading density and kinetic study of immobilized lipase

The activity of the loaded lipase was 738.74 U (~0.01 g lipase powder) /g-lipase-immobilized cocoons. An average of 0.22 g lipase powder was immobilized per gram of dry cocoon. The K_m values (calculated using Lineweaver-Burk plots) of the free and immobilized lipase were found to be 451.26 µM and 257.26 µM, respectively. The reason for the lower K_m value of the immobilized lipase than the free lipase is attributed to the hydrophobic (Kearns et al., 2008; Acharya et al., 2008) and porous (Prasong et al., 2011) nature of the silk-cocoon matrix that provides non-aqueous environment for fast diffusion of the less polar substrates sunflower oil and methanol which resulted effective enzyme-substrate interaction for the catalysis.

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5.3.3 Analysis of biodiesel yield by gas chromatography

In this investigation sunflower oil was selected as the substrate for the transesterification reaction on the basis of the fact that the fuel properties of the methyl ester of the fatty acids obtained from the sunflower oil were nearly similar to those found in the petroleum diesel oil as shown in Table 5.1.

Table 5.1: Comparison of the properties of methyl esters of sunflower oil and petroleum diesel

Property	Sunflower oil methyl ester	Diesel
Specific gravity	0.878	0.835
Calorific value (MJ/kg)	40.56	42.50
Viscosity (mm^2/s) at 40°C	4.5	3.8
Flash point (°C)	85	45
Diesel index	46.9	45.0
Cloud point (°C)	1.0	−1.0

About 1.18 and 1.27 liters of methyl ester of fatty acids obtained from sunflower oil and castor oil, respectively, were required to produce the same energy as that generated by a liter of diesel (Antolin et al., 2002). The analysis of different ester content in biodiesel blends or esterified vegetable oil samples is a unique challenge due to complexity of the sample. However, gas chromatography has been efficiently used for easy analysis of methyl esters (Shashidhara et al., 2003). The major compound formed with both alkali and lipase catalyze transesterification reactions were the esters of palmitic acid (C 16:0), oleic acid (C 18:1), and linoleic acid (C 18:2). Moreover, the ratios of these esters formed in both the transesterification reactions were also found to be nearly same. It is known that the specificity of alkali catalysts for the acyl side chains

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in lipids during transesterification reaction is practically nil (Antczak et al., 2009). Hence, it may be inferred that palmitic acid, oleic acid, and linoleic acid are the predominant fatty acid present as acyl chains in the triacyl glycerol constituent of the substrate sunflower oil used in this investigations. The percentage of linoleic acid, oleic acid, and palmatic acid present in the sunflower oil were ~71.4, ~15.9, and ~ 5.8, respectively (Ihara et al., 1991). We compared the range of product formed during silk-cocoon immobilized lipase catalyzed transesterification reaction with the one formed during conventional alkali catalyzed transesterification reaction in gas chromatograms as shown in figures 5.4 (A) and (B). These findings also further implied that the quality of the methyl esters, and hence, the biodiesel formed during silk-cocoon immobilized lipase catalyzed transesterification reaction would be nearly similar to those obtained by conventional alkali catalyzed transesterification reaction. The conversion yield calculated was based on the relative conversion yield of the three major esters [i.e., the methyl esters of palmitic acid (C 16:0), oleic acid (C 18:1), and linoleic acid (C 18:2)] comparing with the yield obtained by pure alkali catalyzed transeterification of sunflower oil. The yield obtained by using silk-cocoon immobilized lipase was found to be 89%. We calculated the yield by considering that sodium hydroxide catalyzed transesterification of sunflower oil with methanol gives 100% yield. The alkali catalyzed transesterification reaction, was carried out for extended time to ensure complete conversion of sunflower oil to biodiesel. Notably, a biodiesel yield of 97.1% using alkali catalyzed transesterification of sunflower oil has been reported (Umer et al., 2008).

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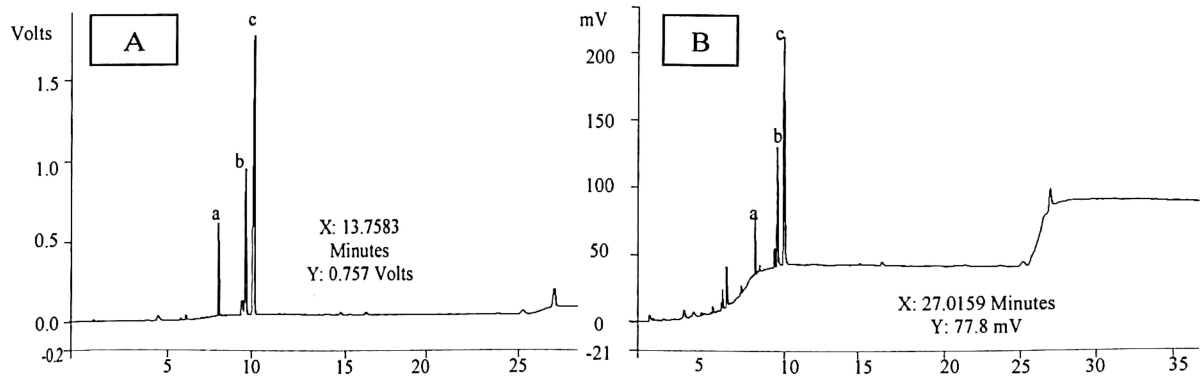


Figure 5.4 Gas chromatogram of fatty acid methyl esters formed in the transesterification reaction catalyzed by [A] chemical catalyst (alkali), and [B] silk-cocoon immobilized lipase. [Methyl esters of a: Palmitic acid (C 16:0), b: Oleic acid (C 18:1), c: Linoleic acid (C 18:2)]

5.3.4 Optimization of alcohol-oil ratio and temperature for transesterification reaction

We studied the substrate conversion yield at different methanol to sunflower oil ratio. The substrate conversion yields (%) of 64.38, 76.86, 78.40 and 76.51 were obtained at the corresponding methanol to sunflower oil ratio (v/v) of 2:1, 4:1, 6:1, and 8:1 (Figure 5.5). Therefore, the ratio of 6:1(v/v) was identified as optimum for the substrate conversion yield at 25°C and 48 hours. The results agreed to the other reports (Freedman et al., 1984; Oliveira and Rosa, 2006). The transesterification reaction was carried out at different temperatures keeping 48 hours as reaction time and methanol: oil ratio at 6:1(v/v). Initially, the ester yield was increased with increasing temperature starting from 20°C. A maximum yield of 89 % was achieved at 37°C (Figure 5.6). Following fifth order polynomial was discerned which fitted well ($R^2 = 0.993$) over the studied temperature range as shown by the smooth curve that coincided with the experimental trend line joining the data points.

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$$Y = -3\times10^{-5}\, t^5 + 0.004\, t^4 - 0.0.317\, t^3 + 10.68\, t^2 - 171.2\, t + 1098,$$

Where, Y is the ester yield and t is the reaction temperature. The influence of temperature on the ester yield during immobilized lipase catalyzed transesterification reaction has been reported by others (Garcia et al., 2002). The thermal stability of the immobilized lipase was comparatively higher than the lipase in its free form; and at elevated temperature beyond 40°C, the yield of the ester was declined.

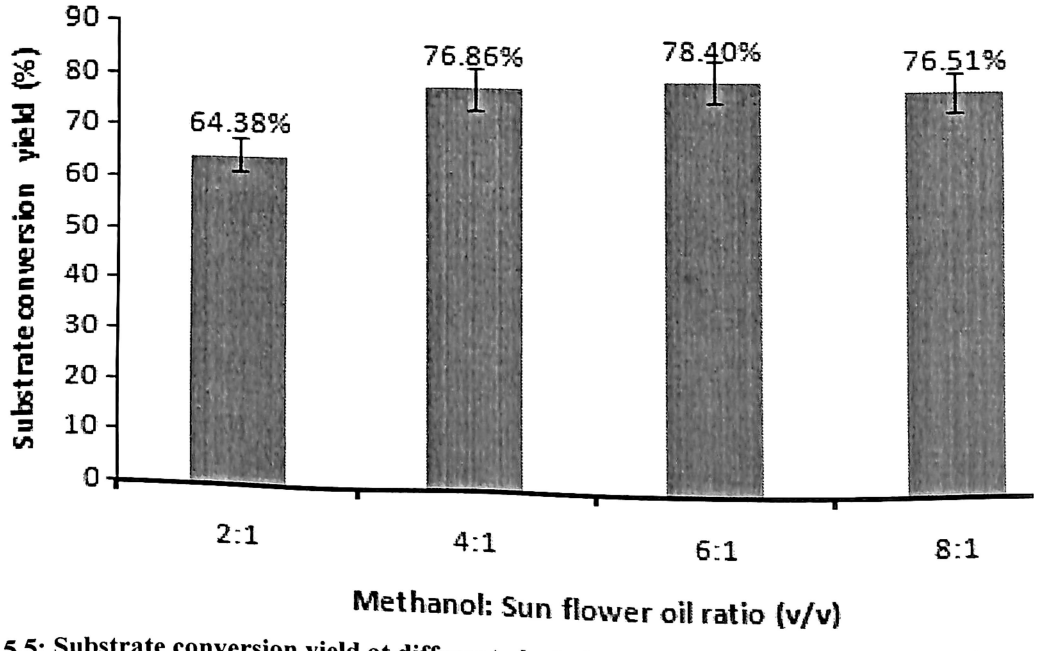


Figure 5.5: Substrate conversion yield at different alcohol: oil ratio during transesterification reaction catalyzed by the silk-cocoon immobilized lipase for 48 hours of reaction [Each datum point represents the average of the analysis of triplicate values represented by error bars]

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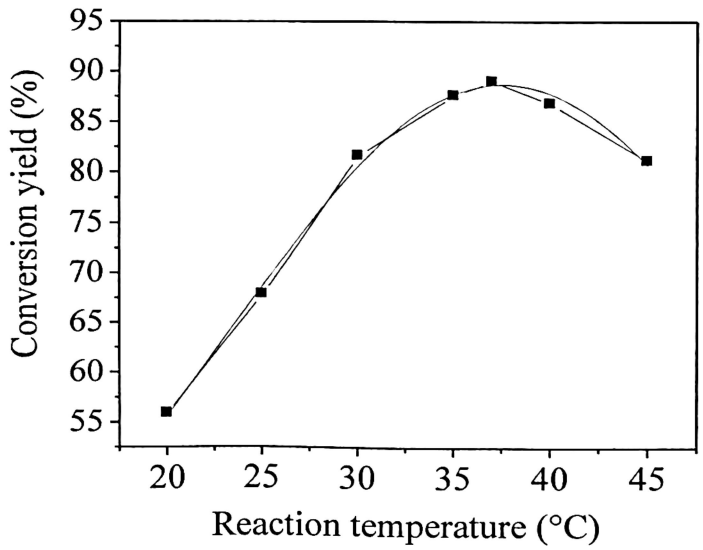


Figure 5.6: Effect of temperature on ester yield during the transesterification reaction catalyzed by the silk-cocoon immobilized lipase in a packed bed bioreactor

[Each datum point represents the average of the analysis of triplicate values]

5.3.5 Biodiesel yield vs. reaction time

The substrate conversion and the corresponding yield of the esters in the bioreactor were monitored at different reaction time intervals at 30°C. The low reaction temperature was chosen to prolong the stability of the enzyme and to reduce the energy consumption involved in the process. The substrate conversion yield reached the stationery phase after 48 hours of the reaction time (Figure 5.7), where maximum conversion achieved was 81.62 %. The conversion pattern followed the following third order polynomial equation with the best fit accuracy of 95.8%.

Substrate conversion % $=10^{-6}\,h^3-h^2+0.028h+0.073$, where 'h' is the reaction time in hour.

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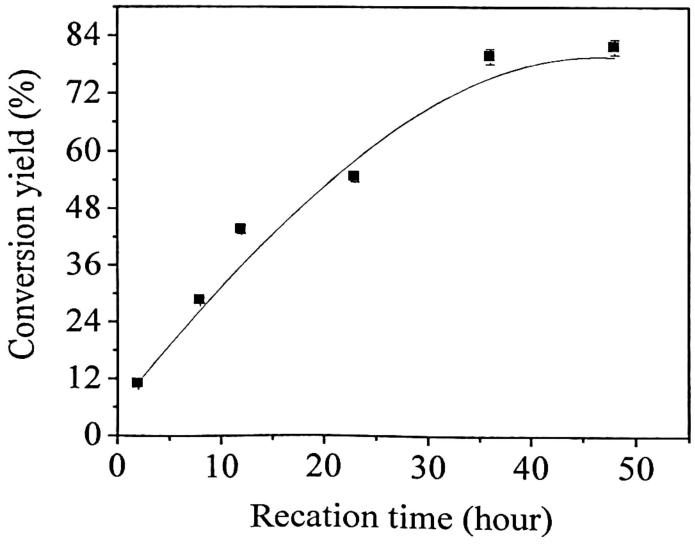


Figure 5.7: Variation in substrate conversion yield during transesterification in packed-bed bioreactor with the progress of reaction time at 30°C [Each datum point represents the average of the analysis of triplicate values]

The reaction was investigated for the consecutive cycles using same set of immobilized lipase keeping 48 hours of reaction time for each cycle (Figure 5.8). The substrate conversion was only marginally decreased till the third cycle of reaction since less than even 5 % of the original activity was decreased in each of the second and third cycle. Nearly 60 % of the original activity was retained in the fourth cycle. At the end of sixth cycle ~37 % of the original activity was retained. The results demonstrated the high operational stability of the silk-cocoon immobilized lipase. The high operational stability of the immobilized lipase would lead to a high cumulative yield of biodiesel by repeating the reaction at least up to the sixth reaction cycle.

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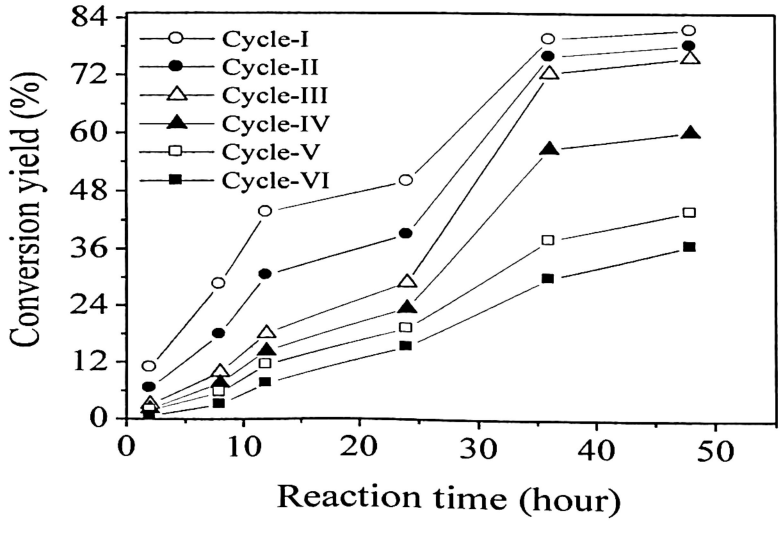


Figure 5.8: Operational stability of the silk cocoon immobilized lipase carried out at 30°C.

5.4 Conclusions

The silk-cocoon, a biodegradable and renewable biomaterial, is demonstrated as novel support matrix for the chemical immobilization of lipase. The cocoon matrix greatly enhanced the catalytic efficiency of the lipase by providing hydrophobic and porous microenvironment that facilitates diffusion of the non-polar substrates to interact with the enzyme and promote the reactions. The immobilization was demonstrated by field- emission scanning electron microscopy and activity study. The lipase loading was 738.74 U (~0.01 g lipase powder) /g-lipase-immobilized matrix. The K_m (Michaelis–Menten constant) of the free and the immobilized lipase was 451.26 μM and 257.26 μM, respectively. Low K_m value of the immobilized lipase is attributed to the hydrophobic nature of the matrix that facilitated the

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substrates diffusion to the enzyme surface. A packed bed bio-reactor was successfully fabricated with the immobilized lipase for transesterification of sunflower oil for the production of biodiesel. The biodiesel yield of 81.62 % was obtained at 48 hours reaction time, 6:1 methanol: oil ratio (v/v) and 30°C. Whereas maximum biodiesel yield of 89% was obtained at 37°C under similar conditions. The conversion yield followed a third order polynomial ($R^2 = 0.95$). The immobilized lipase showed high operational stability at 30°C. The substrate conversion was only marginally decreased till third cycle (each of 48 hours duration) of the reaction since less than even 5 % of the original activity was decreased in each of the second and third cycle. The findings demonstrated the potential of the silk-cocoon as lipase immobilization matrix for industrial production of biodiesel.

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Critical analysis and suggested future scope of the work

The high costs involved in the downstream processing for recovery of product and byproduct, and environmental problems associated with biodiesel production via chemical catalytic methods, have led to the search for alternative methods for industrial production of biodiesel. Enzymatic transesterification of vegetable oil using lipases has attracted much attention for biodiesel production due to its several advantages such as, production of high purity product, easy recovery of the byproduct glycerol, and high yield under environmental reaction conditions. Inspired by the bright prospects of the enzyme catalyzed production of biodiesel, as widely cited in standard literature, we had initiated our work with a *Pseudomonas* lipase considering a report that *Pseudomonas* lipase provides better yield of methyl esters at very higher methanol concentration (96% v/v), as compared to the lipase from the well-known sources such as, *Candida* and *Mucor miehei* (Mittelbach, 1990). The studies on the production of the lipase with various traditional and new substrates have shown burnt oil as the potential substrate for growth and lipase production by the *Pseudomonas* strain. The storage stability of the purified lipase was also found to be very high and comparable only to the few lipases reported in the literature. However, the operational stability observed in the hydrolysis of sunflower oil was poor and specific activity of the purified lipase powder (15800 U/g-protein)

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was not promising to pursue the remaining objectives of the work with this *Pseudomonas* native lipase. The approaches to overcome such problems on stability and activity of the simple enzymes, like lipase, have already been fairly established such as, rational design or *in vitro* evolution (Renugopalakrishnan et al., 2005; Hult and Berglund, 2003) that brings hope to create new enzymes with novel properties for such specific applications. However, the protocols on rational design or *in vitro* evolution involve enormous volume of work and expertise that urged the researchers to take it up separately with an objective focusing primarily on improving the functional properties of the lipase. Hence, we bring in a highly active (73000 Unit/g) commercial lipase prepared from *Aspergillus niger* to augment the work. We observed that the storage stability of the *Aspergillus niger* lipase was also very high as the activity of the lipase was almost retained while kept at 4°C for more than nine months.

The traditional process of hydrolysis of vegetable is clearly energy consuming (requires 3000-5000 kN/m^2 and 250°C) and because of the requirement of high temperatures, the reaction gives rise to a variety of undesirable side reactions, such as polymerization of highly unsaturated fatty acids and production of ketones and hydrocarbons. As a consequence, the resultant fatty acids need to be redistilled to remove byproducts (Gandhi, 1997). Therefore, the lipase catalyzed hydrolysis of vegetable oil is undoubtedly an approach bearing better perspective, owing to the requirement of low temperature for the reaction as demonstrated through this investigation. The reason for high catalytic activity of the lipase, for the hydrolysis of surfactant mediated emulsion, is attributed to the phenomena of interfacial activation of the lipase catalyst. However, the enhanced catalytic activity of the lipase in the biosurfactant mediated hydrolysis of the oil is intriguing. Further investigation on the interfacial microenvironment created by the biosurfactant between the lipase molecules and the substrates phase is warranted to reveal the fact.

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The nature of the immobilization support material is very important for the efficiency of the lipase and other enzymes as well. The immobilization may also prevent the lipase from deactivation by the organic substrates (alcohols in the present case) and the byproducts produced during the enzyme-catalyzed reactions. Generally, hydrophilic supports tend to compete with enzyme for the available water in the environment that lead to a higher water concentration in the environment of the enzyme favoring hydrolytic reactions. Most widely used supports are ion exchange resins, celite and biopolymers such as Accurel EP100 (Lee and Akoh, 1998). We have eventually selected silk as immobilizing support material for the lipase visualizing the advantages of these biomaterials such as, (a) biocompatibility that support stability to the lipase, (b) structural protein that prevents its dissolution or leaching to the reaction medium, and (c) hydrophobic nature that support better sequestration of the amphipathic and less-polar substrate (the vegetable oil and co-substrate methanol in the present case) to the catalytic site of the lipase. The silk we used initially for lipase immobilization for hydrolysis of sunflower oil was planner in geometry (as it was in the mat form); the same configuration of the support did not give promising results when used in the bioreactor designed for the transesterification reaction to produce the biodiesel. The reason is ascribed to the fouling caused by the low pore sizes in the mat and viscous sunflower oil substrate that cause fouling and linked high back pressure in the reactor, which eventually reduced the efficiency of the reactor. The geometry of the support is obviously very important for the efficiency of the immobilization. It is known that large pores (> 100 nm) are required for the efficiency (activity/loading) to become independent of pore diameter, below this value, the enzyme activity was highly dependent on pore diameter, as a result of limitation in the rate of diffusion of substrate. To maintain the required porosity of the immobilizing silk material in the reactor we had used uniformly chopped desericinated silk-

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cocoons directly as lipase immobilizing support for the production of the biodiesel. The result, as evident from the level of substrate conversion and product yield, was highly promising. One of the findings that attracted our attention most is the effect of the silk-cocoon on lowering the Michaelis–Menten constant (K_m). The reason for the reduction of K_m value is attributed to the porous and hydrophobic nature of the silk-cocoon, which promotes the non-aqueous environment for rapid diffusion and transesterification of the amphipathic substrate. The strong rigid structure of the silk-cocoon is very likely to support high porosity (as is also visible from the microscopic images) of the lipase immobilized matrix. Through this preliminary investigation we forward the new concept of silk-based material as lipase immobilizing support for lipid hydrolysis and biodiesel production. Further in-depth investigation on the effect of geometry of the silk-cocoon, the reactor design, the kinetics of the transesterification reaction at various flow rates of the substrate and temperature will obviously help to develop a potential lipase catalyzed-based production of biodiesel for commercial application.

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Published

- **Chatterjee S.**, Barbora L., Cameotra S. S., Mahanta P., Goswami P.* (2009) **'Silk-fiber immobilized lipase-catalyzed hydrolysis of emulsified sunflower oil'**, *Journal of* Applied Biochemistry & Biotechnology 157(3): 593-600.

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Under review

Chatterjee S., Yadav D., Barbora L., Mahanta P., Goswami P. * **'Silk-cocoon matrix immobilized lipase catalyzed transesterification of sunflower oil for production of biodiesel'.**

Book Chapter

Chatterjee S., Barbora L., Kumar A. K., Goswami P., Dutta N. N., Goswami P. **"Purification, immobilization and application of a *Pseudomonas* lipase for transesterification of lipid for production of biodiesel from vegetable oil"**. Biodiesel. Production. Technologies. 1st ed. Jaipur, Pointer publishers. (2007). xviii, 206 p. ISBN: 8171325177.

Papers presented in Conference

Chatterjee S., Barbora L., Kumar A K., Goswami P., Dutta N. N., Goswami P. * **'Purification, immobilization and application of a *Pseudomonas* lipase for transesterification of lipid for production of biodiesel from vegetable oil'** in oral session of International conference on 'Biofuel Vision 2015' at Engineering College Bikaner on October 15, 2006. Mr. Chatterjee was honored for the best presentation in that conference. This news appeared in newspaper 'The Hindu' October 16, 2006 (page 3).

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