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Transesterification of used sunflower oil using immobilized enzyme

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ABSTRACT

Transesterification reaction was performed using used sunflower oil and short-chain alcohol by immobilized lipase in non-aqueous conditions. The long-chain fatty acid alkyl ester, which is the product of this reaction, can be used as a diesel fuel that does not produce sulfur oxide and minimize the soot particulate. Immobilized enzymes Lipozyme RM IM and Novozyme 435 were screened. Lipozyme RM IM showed the highest activity in this reaction with mild reaction temperature of 45 °C. In solvent free system, there is inactivation of enzyme by methanol and glycerol. Hence, tertiary butanol is found to be a good solvent which solubilizes both triglycerides and glycerol. The activity of immobilized lipase was highly increased in comparison with solvent free system because its activity sites became more effective. Immobilized enzyme could be repeatedly used without troublesome method of separation and the decrease in its activity was not largely observed.

1. Introduction

Petroleum fuel is an important fuel used for many purposes in the world of which reserves are vanishing gradually as well as emission of harmful gases due to burning of these fuels creates environmental pollution. Thus, there is need to find alternative for these fuels. The substitution of conventional fuels (gasoline, diesel) by renewable biofuels is considered a potential way to reduce pollution and to support the sustainable development of the country. With the current consumption level of about 85 million barrels per day of oil and 260 billion cubic feet per day of natural gas, the reserves represent 40 years of oil and 64 years of natural gas [1]. India is consuming over 127 million tones of crude oil per year and is forced to import about 70% of its need. In India, current consumption of diesel alone is approximately 40 million tones per year constituting about 40% of all petro-products [2]. Biodiesel derived from the plant (bearing oils) like sunflower, rapeseed, canola or jatropa curcas can be used as a substitute or an additive to diesel. Biodiesel can provide power similar to conventional diesel and thus can be used in diesel engines. Biodiesel is a non-toxic and environmental friendly as it produces substantially less CO and the combustion gases contain no sulphur dioxide and unburnt hydrocarbons.

The use of 100% pure vegetable oil or animal fats to power diesel engines has several drawbacks such as high fuel viscosity, low power output, thickening or gelling of lubricating oil and low volatility resulting in carbon deposits due to incomplete combustion. Hence, vegetable oils are processed so as to acquire properties (volatility and viscosity) similar to that of fossil fuels [3]. Although, various processing techniques like pyrolysis, microemulsification and transesterification are available to convert vegetable oil to fuel form, transesterification is the most popular method of producing biodiesel. Currently biodiesel is most commonly prepared by alkali/acid catalyzed transesterification of an oil or fat with an alcohol, or acid catalyzed esterification when the feedstock has high free fatty acid content [4]. Chemical methods give high conversion ratio of triacylglycerols (TAG) to methyl esters (Biodiesel) in short times (4–10 h). However, chemical transesterification are connected with some drawbacks as for example, high energy consumption and difficulty in the recovery of glycerol and high amount of alkaline waste water from the catalyst [5]. Enzyme catalyzed transestserfication of oil is a good alternative to overcome these drawbacks.

There are many reports on biodiesel production using enzyme catalysis by free or immobilized lipases [6]. Immobilized lipase in particular is suitable for continuous biodiesel production because of the ease of its recovery from the reaction mixture. There are two major limitations of lipase-catalyzed biodiesel synthesis. One is higher cost which can be reduced up to a certain extent by immobilization [7] and another is its inactivation by methanol and glycerol [8]. It has been reported that as methanol is insoluble in vegetable oils, it inhibits the immobilized lipases and thereby decreases the catalytic activity of the transesterification reaction. Further, the hydrophilic by-product glycerol is also insoluble in the oil, so it is easily adsorbed onto the surface of the immobilized lipase leading to a negative effect on lipase activity and operational stability

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Table 1
Properties of used frying oil and refined sunflower oil.

Properties	Used frying oil	Refined sunflower oil
Linoleic acid (%)	53.4	52.78
Oleic acid (%)	35.34	35.71
Palmitic acid (%)	9.08	3.73
Stearic acid (%)	2.16	7.76
Saponification value (mg KOH/g of oil)	208	192
Density (kg/m^3)	925	890
% FFA (mg KOH/g oil)	2.805	0.706

[9]. Use of several solvents such as n-hexane and petroleum ether in the reaction medium has been reported [8,10,11] but the problem persisted since the inhibition of lipases still occurred due to poor solubility of methanol and glycerol in the hydrophobic solvents. There are some reports on enhanced biodiesel synthesis in presence of tertiary butanol as a solvent [12]. As both methanol and glycerol are soluble in tertiary butanol, the inhibitory effect of methanol and glycerol on lipase activity is reduced. Moreover, t-butanol is not a substrate for the lipases because it does not act on tertiary alcohols [13].

As the cost of the biodiesel depends largely upon on feedstock, its selection is very important. Different feed stocks available for transesterification includes edible vegetable oils, e.g., soybean oil [14], safflower oil [15], rapeseed oil, cottonseed oil [12,16], non-edible oil, e.g. *Jatropha oil*, animal fats, sunflower oil [17–20] and waste oils, e.g., used frying oils [21,22]. A lot of research has been carried out with refined vegetable oil as feed stock for transesterification, however used frying oil which is less expensive than pure vegetable oil is a promising alternative to vegetable oil for biodiesel production. Thus in the present work, optimization of transesterification of used sunflower oil using immobilized lipase enzyme has been carried out. The main aim was to check the application of the immobilized enzyme for the transesterfication of used sunflower oil.

2. Materials and methods

2.1. Materials

Novozyme 435 (lipase B from *Candida antarctica*; immobilized on macro-porous polyacrylic resin beads, bead size 0.3–0.9 mm, bulk density 0.430 g/cm^3) and Lipozyme RM IM (*Mucor miehei* lipase immobilized on anionic resin, size 3–6 mm, density 0.75 g/cm^3) were procured from Novo Nordisk A/S Bagsvaerd, Denmark.

Used frying oil was purchased from Garnish restaurant, King's Circle, Mumbai. The refined sunflower oil was supplied as a gift sample by the same restaurant. The used frying oil is composed of 90% of the unsaturated fatty acids (linoleic and oleic acids) and 10% saturated fatty acids (palmitic and stearic acid). Table 1 shows the properties of both used frying oil and refined sunflower oil. This oil was heated at 180–190 °C for around 5–6 h for frying the vegetable food. During frying polymers are formed and hence, there is change in the properties of both the oils.

Methanol (99%), n-butanol, tertiary butanol, oleic acid, iso-octane, potassium hydroxide, sodium hydroxide, hexane, heptane, petroleum ether etc. used in the experimental work were purchased from S. D Fine-Chem. Ltd., Mumbai. Acetonitrile and acetone (HPLC grade) used as solvent for HPLC analysis were purchased from G. Kuntal Implements, Mumbai. Methyl oleate and Methyl linoleate were purchased from Sigma–Aldrich.

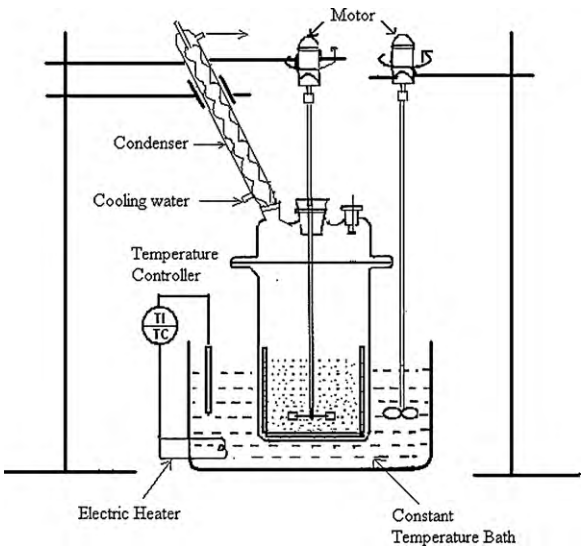

Fig. 1. Experimental setup for the transesterification reaction.

2.2. Experimental method

The experimental setup consisted of a 4.5 cm i.d. glass reactor of 100 ml capacity, equipped with six-bladed turbine impeller. The entire reactor assembly was immersed in a thermostatic water bath, which was maintained at the desired temperature with an accuracy of ±5 °C. The reaction temperature was monitored with the help of a temperature controller. The reactor was also equipped with a condenser to reduce the losses of methanol due to evaporation. The agitation was provided by means of an electric motor having provision for speed control. The mixture of used frying oil and methanol was first fed to the reactor and stirred at known RPM. After attainment of the desired temperature, the immobilized enzyme of known quantity was added to it. Samples from the reaction mixture were drawn at regular intervals of time. Samples of the reaction mixture were centrifuged to remove immobilized lipase before analysis. The schematic of experimental setup is shown in Fig. 1.

2.3. Analytical method

2.3.1. Fatty acid composition and fatty acid methyl ester

The fatty acid compositions of triacylglycerols were determined by converting all fatty acids of triacylglycerols into the corresponding fatty acid methyl esters followed by gas chromatography (GC) analysis using BP-X70 column. Fatty acid methyl esters content in the reaction mixture were analyzed on HPLC using octadecyl (C18) 5 μm, length 4.6 mm × 250 mm column at 210 nm. The samples were analyzed isocratically using acetonitrile:acetone (90:10) with 1 ml/min flowrate. The used sunflower oil in this study is composed of 90% of the unsaturated fatty acids (linoleic and oleic acids) and 10% saturated fatty acids (palmitic and stearic acid).

2.3.2. Esterification activity of enzyme

Esterification activity of an immobilized lipase was estimated through an esterification reaction. 200 mg of vacuum dried immobilized lipase material was added to screw capped vial containing a mixture of 0.32 ml oleic acid and 0.27 ml dry n-butanol in 3 ml dry iso-octane and 0.05 ml distilled water. The vials were placed in a controlled temperature shaker at 30 °C and shaken at 250 rpm. The reaction was stopped by addition of 10 ml of methanol and the reaction was immediately titrated for unreacted fatty acid against 0.05 M alcoholic NaOH using phenolpthalein indicator. Immobi-

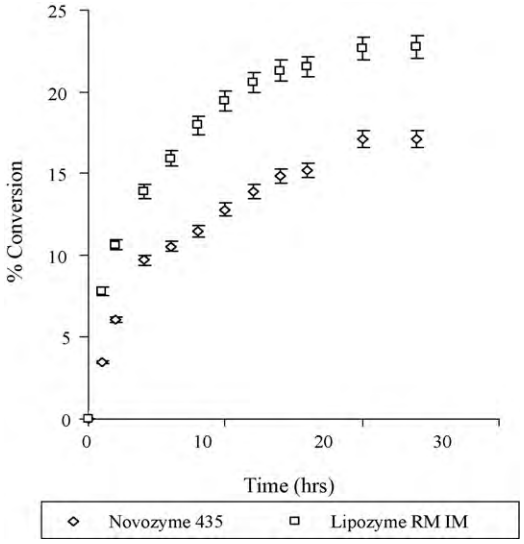

Fig. 2. Screening of enzyme.

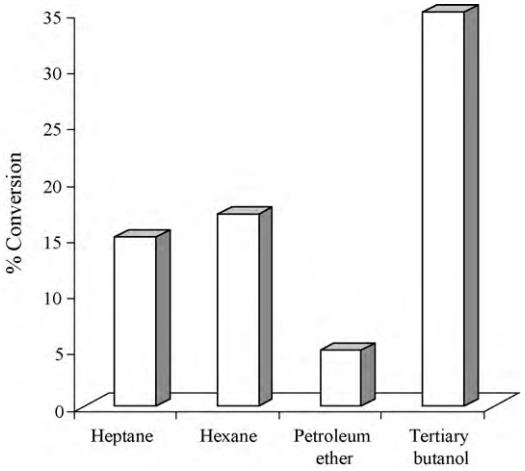

Fig. 3. Effect of solvent on % conversion.

lized esterification activity was expressed as number of μmoles of ester formed per min reaction conditions.

The esterification activity was determined by the following correlations:

$$\text{Esterification activity Ea} = \frac{V \times M \times 100}{E \times T}$$

where V: difference in volume in ml of NaOH between the blank and samples after time T (period of incubation in min); which is a measure of oleic acid consumed during esterification; M: molarity of NaOH; E: amount of enzyme employed in mg.

One unit of enzyme activity is defined as 1 μmole of oleic acid consumed in the esterification reaction per min per mg lipase.

3. Results and discussions

3.1. Screening of enzyme

Lipases were screened for their ability to transesterify triglycerides with short-chain alcohols to alkyl esters. Two commercially immobilized lipases namely, Lipozyme RM IM and Novozyme 435 were screened to find the % conversion in the transesterification reaction of used frying oil and the results are showed in Fig. 2. It was found that the conversion obtained after 24 h using Lipozyme RM IM was 22% as compared to Novozyme 435 which showed 17% conversion. Lipozyme RM IM was used for the further study. This is because lipase from *Mucor miehie* (Lipozyme RM IM) which is 1,3-specific lipase was most efficient for converting to their alkyl esters with primary alcohols, whereas the lipase from *Candida antarctica* (Novozyme 435) was most efficient for transesterifying triglycerides with secondary alcohol to give branched alkyl esters [23]. Also the esterification activity of Lipozyme RM IM was found to be 3.3 U/g as compared to 2.9 U/g for Novozyme 435.

3.2. Effect of solvent

In solvent free system, the conversion of Lipozyme RM IM is less, i.e. 22% because of the inactivation of enzyme by methanol and the byproduct glycerol which is adsorbed on the external surface of enzyme. With the addition of solvents viz. hexane, heptane, petroleum ether and tertiary butanol this conversion could be increased [24]. Transesterification reaction of used frying oil using Lipozyme RM IM in these organic solvents showed that FAME conversion is high in hydrophilic solvents; tertiary butanol, i.e. 35% but low in hydrophobic solvents; heptane (15%), petroleum ether (5%) and hexane (17%) as shown in Fig. 3. According to Halim and Kamaruddin [25] which states that lipase has high synthesis activity and good stability in hydrophobic solvents like hexane which is contradicting with the present results. Hydrophilic compound used as substrate (methanol) or obtained product (glycerol), are immiscible in hydrophobic reaction medium. The glycerol deposit which was adsorbed on the Lipozyme RM IM during this process is expected to reduce the lipase activity. Methanol is insoluble in the mixture of oil and hydrophobic solvent (e.g., hexane, petroleum ether and heptane). Hence, % conversion is less in these solvents. The byproduct glycerol which is a hydrophilic in nature has poor solubility in these relatively hydrophobic solvents. Even though the methanol is easily soluble in the hydrophilic solvent (acetone, acetonitrile), but the oil is immiscible in these hydrophilic solvent and will also lead to low FAME conversion. Tertiary butanol which is a moderately hydrophilic solvent can solubilize oil, methanol and glycerol. It is not a substrate for lipases because it does not act on tertiary alcohols [12]. Tertiary butanol solvent therefore improves the enzyme catalytic properties leading to higher fatty acid methyl ester conversion. These results are in agreement with Royon et al. [12] and Wei et al. [13].

3.3. Effect of temperature

As enzymes are very sensitive to temperature, the effect of temperature on the transesterification reaction was studied. From reaction temperature 25–45 °C, the initial rate as well as the conversion was found to increase from 24% to 40% with an increase in temperature but at 55 °C, the initial rate was nearly same as that of 45 °C but the final conversion was decreased, i.e. 36% (Fig. 4). This is because thermal denaturation of the enzyme takes place at higher temperature which leads to the decrease in conversion. After completion of the reaction, the enzyme was washed and its esterification activity was determined. From Fig. 5, it can be seen that the activity remains constant up to 45 °C (3.12 U/g) but at 55 °C there is decrease in activity (2.625 U/g) due the thermal denaturation. Therefore, the optimum temperature selected was 45 °C, since it was the least temperature at which maximum conversion was obtained.

3.4. Effect of speed of agitation

In the case of immobilized catalysts, the reactants have to diffuse from the bulk liquid to the external surface of the particle and

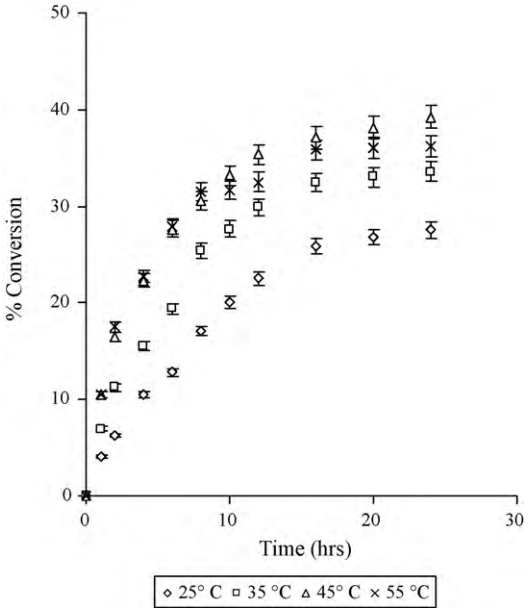

Fig. 4. Effect of temperature on % conversion.

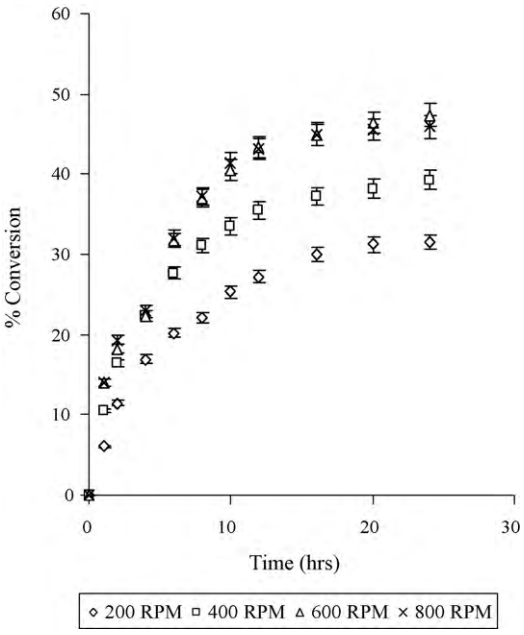

Fig. 6. Effect of speed of agitation on %conversion.

from there into the interior pores of the catalyst. External mass transfer and internal diffusion limitations can be minimized by carrying out the reaction at an optimum speed of agitation and low enzyme loading of optimum particle size, respectively. The effect of speed of agitation on the % conversion was studied in the range of 200–800 RPM. From Figs. 6 and 7, it was seen that both rate and conversion increases from 32% to 47% by increasing speed from 200 to 600 RPM. However, at 800 RPM, rate of the reaction remains constant (0.0025×10^2 (kmole/m^3/h)) as shown in Fig. 7. This is because from 200 to 600 RPM, the reaction is mass transfer controlled and beyond 600 RPM the reaction is kinetically controlled. Thus, optimum speed of agitation was found at 600 RPM. At 800 RPM, it was seen that there was slight decrease in conversion due to the shearing of the lipase molecule or inactivation of the lipase. It was also observed that, some immobilized lipases were not in the liquid phase but it stuck to the wall of the reactor due to the high speed thereby reducing the effective enzyme loading in the reaction mixture. According to Wiez Prater criterion, if C_{wp} is less than one then the reaction is surface reaction controlled and if it is greater than one then there is internal diffusional resistance. Therefore, to rule out intra-particle diffusional resistance, Wiez Prater criterion [26] was followed and it was found that $C_{wp} = 3.098 \times 10^{-3}$, which is less than one. Hence, the reaction is surface reaction controlled.

3.5. Effect of enzyme loading

The amount of enzyme used is a crucial economical factor for successful industrial application. Therefore, the effect of enzyme loading was examined. To study this effect, the % enzyme loading was increased from 0.5% to 6% (w/w) with respect to oil. Reaction temperature was fixed at 45 °C and methanol to oil ratio was 3:1. From Fig. 8, it is seen that there was an increase in the FAME conversion from 27% to 56% by increasing the lipase quantity 0.5–4%. However, further increase in the lipase quantity of 6% did not have that much effect on FAME conversion. This suggested that the amount of enzyme added was much greater than required and external mass transfer resistances had limited the rate. Also, the addition of larger lipase quantity was not practical since the matrix and lipase together made the solution extremely viscous and there

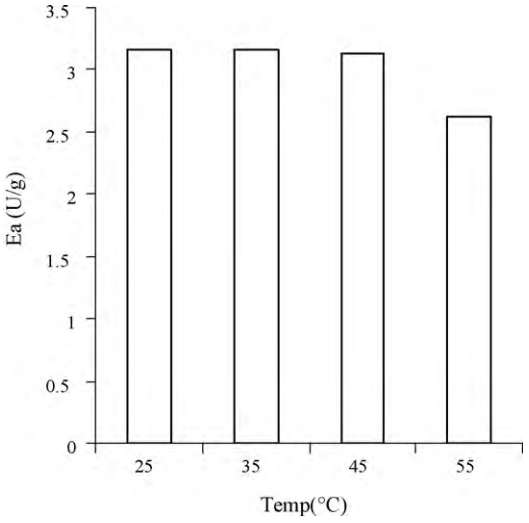

Fig. 5. Effect of temperature on the activity of enzyme.

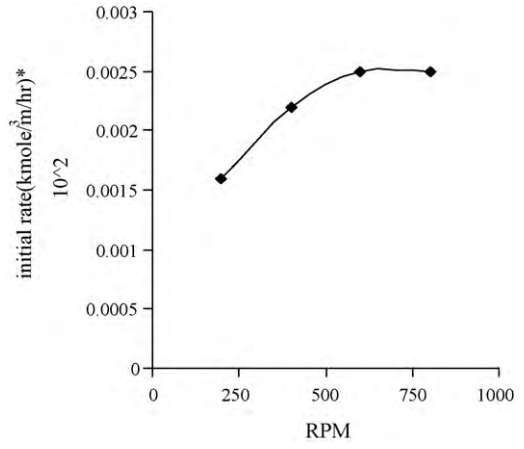

Fig. 7. Effect of speed of agitation on the initial rate of reaction.

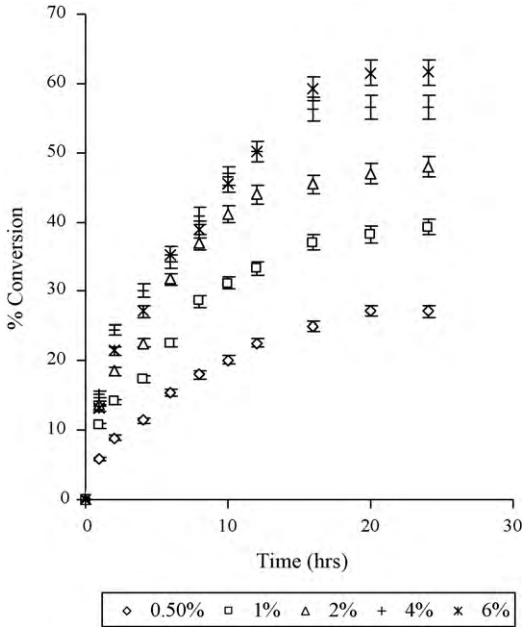

Fig. 8. Effect of enzyme loading on %conversion.

was no significant increase in % FAME conversion. Considering the economical point of view and also the % conversion, 4% enzyme loading was used in the further study.

3.6. Effect of methanol to oil molar ratio

The effect of oil to methanol molar ratio on % conversion of oil was studied as it is an important parameter in the enzymatic transesterification reaction. Experiments were conducted with different molar ratios of methanol to oil ranging from 3:1 to 7.5:1. In the transesterification reaction as per stoichiometry, three moles of methanol reacts with one mole of triglycerides to yield three moles of fatty acid methyl esters and one mole of glycerol. Results obtained are as shown in Fig. 9. The FAME conversion increased from 3:1 to 4.5:1 with increase in conversion from 56% to 66%. Beyond 4.5:1, the FAME conversion was 67% showing no significant increase, thus 4.5:1 ratio was considered as optimum. From Fig. 10, the esterification activity of the enzyme states that there is decrease in activity from 3.29 to 2.66 U/g because of the deactivation of enzyme by methanol at higher molar ratio.

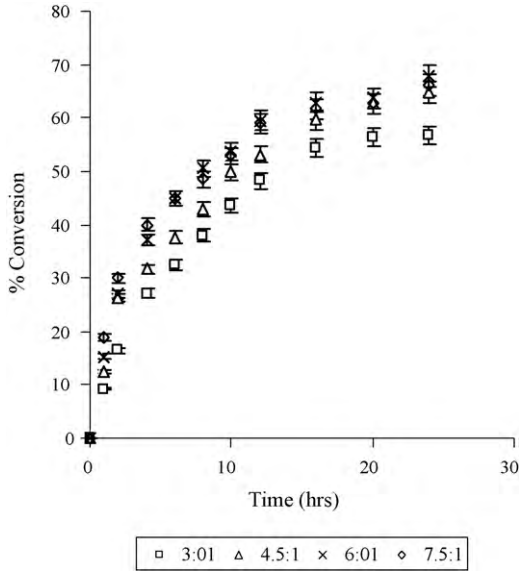

Fig. 9. Effect of methanol to oil molar ratio on %conversion.

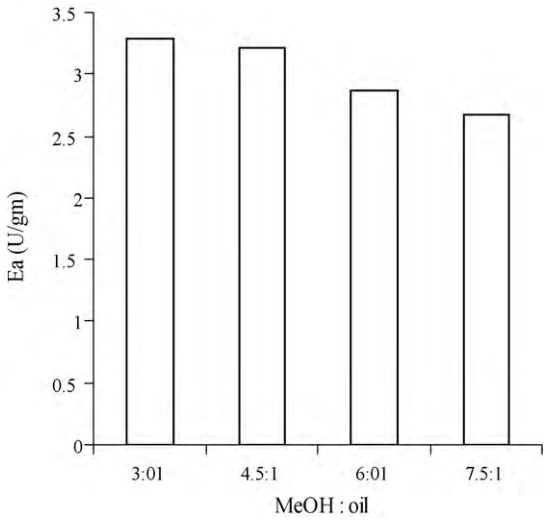

Fig. 10. Effect of methanol to oil molar ratio on the activity.

3.7. Reusability of enzyme

The optimized parameters were used to check the reusability of enzyme. After completion of the reaction, the immobilized enzyme was filtered off, washed with acetone and reused for 6 times as shown in Fig. 11. It was found that the transesterification activity remained unaffected even after the sixth reuse and conversion remains more or less the same.

3.8. Comparison of used frying oil with refined sunflower oil

Transesterification of refined sunflower oil was also explored. From Fig. 12, it is seen that the trend for used frying oil and the

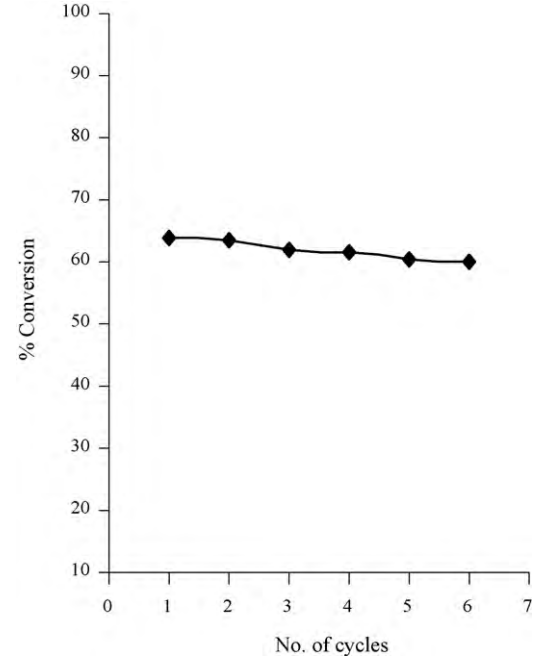

Fig. 11. Reusability of the enzyme on %conversion.

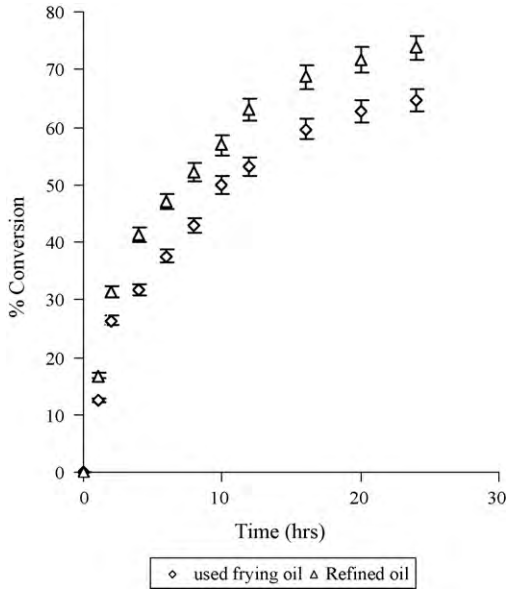

Fig. 12. Comparison of used frying oil and refined oil.

refined sunflower oil is almost the same. The highest FAME conversion was achieved for refined oil as compared to used frying oil. It was observed that used frying oil has slightly higher water content compared to refined oil. Water is known to have inhibition effect on lipase activity in the transesterification reaction. At high water content, diffusive limitations of the substrate can occur and water can promote the hydrolysis of the substrate thus decreasing the conversion of the product.

4. Conclusion

Biodiesel production of used sunflower oil was successfully carried out in tertiary butanol system as the reaction medium. Lipozyme RM IM was found to be a suitable catalyst for this process. It has also been demonstrated that 4.5:1 methanol to oil molar ratio does not inhibit the Lipozyme RM IM in this tertiary butanol system. Tertiary butanol alters the mechanism of lipase-catalyzed biodiesel production, presumably by causing the conformational change on the structures of lipases, which in turns eliminates inhibition of enzymatic activity by alcohol. The reusability characteristic states that the activity of the enzyme remains constant after the 6th reuse with conversion remains more or less the same.

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