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Research Article

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Analysis of reaction parameters for the transesterification of algal oil from *Enteromorpha compressa* using purified lipase and whole cell biocatalyst

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ABSTRACT

The latest developments in the production of biodiesel explores the feasibility for utilization of marine algal oil as a source. In this investigation, the focus is laid on the transesterification of algal oil obtained from marine macroalgae Enteromorpha compressa using biochemical catalysts- Purified Lipase and Whole cell (Rhizopus oryzae 262). Optimization of reaction parameters including temperature, catalyst concentration, oil to alcohol ratio, rate of stirring, time and pH was carried out independently for both catalysts with methanol and further the comparative analysis was extended to another six alcohols (Ethanol, Propanol, Butanol, iso-propanol, iso-butanol and iso-amyl alcohol) for which the yields were analysed at optimized reaction conditions. Maximum yield obtained for purified lipase was 92% for methanol under the conditions of 30 °C, 12.5%, 1:3, 150 rpm, 24 h and 7 ph. Maximum yield obtained for whole cell biocatalyst was 83% for methanol under the conditions of 30 °C, 50 beads, 1:3, 175 rpm, 48 h and 6 pH. Comparative analysis of yields for various alcohols showed that purified lipase was better as a catalyst. Results can be used to produce fourth generation (Algal) biodiesel in a consistent and sustained manner.

Keywords: Biodiesel, Biocatalysis, Lipase, Optimization, Immobilized Cells, Algae

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INTRODUCTION

Uninterrupted diminution of fossil fuels due to incessant fossil based energy practices has landed the planet in alert of immediate fuel demands. Alternate fuel was the fervently focused subject for the past few decades since the use of fossil energy resulted in alarming rise of gas particulates. Sustainable energy supply with reduced release of toxic gases was the providential characteristic of biofuels. Apart from being less toxic, a fuel that is to replace the current fossil fuel must have these characteristics as well- Available throughout the year, Consistent in quality and must meet standards. In the case of biodiesel, the main factors that govern above mentioned characteristics are the source of raw material and production process [1].In the past several sources were tried as a potential replacement for conventional fossil fuel. Researchers have used both the crude vegetable oils and the ester forms of the oils [2]. Even though the crude form failed to have an impact, the ester form of the oils or fatty acids showed convincing results that matched the performance of diesel in engines. The esterified oils are called as Fatty Acyl Alkyl Esters or simply biodiesel. Earlier researches focused on the use of readily available vegetable (Edible and non-edible) oils for biodiesel production [3, 4] which was later expanded to the use of waste cooking oil [5]. Both these sources suffered

from the drawback of uneconomical cultivation process and irregularities in availability. The present generation of research is focused on the use of microalgae [6] as an oil source and has been extended to the exploitation of marine macro algae [7, 8] for its feasible growth conditions and oil production as compared with microalgae.The next factor for consideration in obtaining quality biodiesel is the process involved in the conversion of the oil into biodiesel. The chemical process of conversion of oil into Biodiesel is called as transesterification and it can be divided into catalyst mediated and non-catalyst mediated. Non-catalyst mediated or super critical methanol method for transesterification is superior and fastidious but lacks in the knack for commercialization in the present [9]. The viable option for transesterification in the present scenario is in the use of either of the three catalysts- Acid [10], Alkali [11] and Biocatalysts [12, 13]. Many homogenous and heterogeneous acid and alkali catalyst have shown significant yields of biodiesel with appreciable reaction rates. The main drawbacks of this type of catalysis is the requirement of large amount of energy and acyl acceptor, inability to transesterify oil containing high free fatty acids and to produce clean by-products[14]. Biocatalysts such as lipase or whole cell biocatalyst counter all the above problems at the cost of slower reaction rates [15].

The present work focuses on utilizing *Enteromorpha compressa*, a species of marine macrolagae belonging to ulvaceae family which is found prominently in Indian coastline as the source of oil feedstock. The transesterification process is to be done using biocatalysts- purified enzyme lipase and whole cell biocatalyst preparation of *Rhizopus oryzae* 262. Whole cell biocatalyst of *Rhizopus oryzae* has been shown to produce 1,3-position specific lipase [16] which is to be utilized for catalysis. Hence in this work both the biocatalyst preparations of immobilized purified lipase and immobilized whole cell biocatalyst of RO 262 is used to evaluate for their potential to transesterify algal oil. Further, optimization of various reaction parameters and catalysis with different acyl acceptors was done.

MATERIALS AND METHODS

2.1. Materials

Organic solvents including Hexane, Methanol, Ethanol, Propanol, Butanol, Iso-propanol, Iso-butanol and Iso-amyl alcohol of industrial grade was obtained from Merck Limited, Mumbai, India.

2.2. Collection and preparation of algal sample

Enteromorpha compressa was collected from Gulf of Mannar, Mandapam coast, Rameswaram, India (Lat: 9° 16' 52.7658" and Long: 79° 18' 55.1268"). The macro algal sample was washed with distilled water, dried in shade condition for few days, then was sieved and pulverized into uniform sized particles. Humidity content (%) of the algal sample was determined by drying 20g of sample in a thermo regulated incubator (Memert 40050ip20, Germany) at 105°C until a constant weight was reached [17]. Calculation of the humidity content was done using the following formula,

$$Humidity\ Content = \frac{P_a - P_f}{P_a} \times 100 \quad (1)$$

Where, P_a is the initial weight of the sample and P_f is the final weight of the sample after drying. Taking into account that water activity (which is the water not bound to the system and can be evaporated) influences the solvent extraction and lipase activity, humidity content was determined and reduced to optimum [18].

2.3. Extraction and treatment of algal oil

The pulverized algae after dehumidification was subjected to ultrasonication at 24 kHz with constant temperature (50 °C ± 1) and for a time interval of 5 minutes. Ultrasonication enhanced the net amount of oil obtained. The algae after ultrasonication was directly loaded into the Soxhlet extractor and the solvent extraction was done for each batch using hexane for 3 hours [19]. The amount of solvent was maintained to five times of the amount of the algal mass. The solvent mixture was removed from the sample extracts using the rotary vacuum evaporator and the excess solvents were reused for following batches. The algal oil was filtered using Whatman filter paper No.42 for removing crude impurities and the filtrate was stirred with 1% of 85 % phosphoric acid for 10 minutes using a magnetic stirrer at 80°C, followed by mixing with sterile distilled water for 30 minutes. The mixture was then allowed to settle in a separating funnel and the oil was separated from other activated constituents. The obtained oil was then treated with activated charcoal to remove coloured impurities.

The mass percentage of the total lipid extracted was calculated from the difference in the flask weight at the start and end of the extraction process. The quantity of lipid extracted from the sample was calculated using the formula,

$$Oil\ extraction\ yield\ (\%) = \frac{Weight\ of\ oil\ extracted\ (g)}{Weight\ of\ algal\ biomass\ (g)} \times 100 \quad (2)$$

2.4. Biocatalyst preparation

2.4.1. Lipase

The pure enzyme lipoprotein lipase was purchased from SISCO research laboratories Private., Limited., Mumbai. Lipase was immobilized by entrapment immobilization using calcium alginate. Calcium alginate beads were prepared using 2% of Sodium alginate solution in distilled water and calcium chloride of 0.1 M. Immobilization procedure was carried out for the enzyme and it was observed that larger the surface area of the beads and lower the concentration of the sodium alginate more is the number of pores on the surface. As the concentration of the sodium alginate increased, the thickness of the beads also increased, there by indirectly decreasing the number of pores.Pre incubation of the immobilized lipase was observed to increase the initial rate of catalysis. Therefore, the immobilized lipase was incubated in methyl oleate for 30 minutes followed by incubation in the algal oil for 12 hours. The extent of decrease in reaction rate caused by water was reduced by pre incubation as the pores of the support matrix were occupied by methyl oleate and algal oil.

Enzyme activity in terms of the amount of enzyme which releases one mille equivalent of free fatty acid per minute per gram of triglyceride sample was determined using the titration with 0.1N NaOH of free fatty acids produced during the lipase catalysis.

$$Activity\ \left(\frac{meq/min}{gram} of\ sample\ \right) = \frac{Volume\ of\ alkali\ consumed \times Strength\ of\ alkali}{Weight\ of\ sample\ (g) \times Time\ (min)} \times 100 \quad (3)$$

2.4.2. Whole cell biocatalyst

Rhizophus oryzae a filamentous fungi was obtained from MTCC (Microbial Type Culture Collection Centre, Chandigarh) and used as whole cell biocatalyst for the conversion algal oil into biodiesel. In order to obtain maximum catalysis, the fungi was cultivated in a special culture medium containing 30 g/l of refined olive oil, 70 g/l peptone, 1.2 g/l $NaNO_3$, 1.2 g/l KH_2PO_4 and 0.5 g/l $MgSO_4.7H_2O$. The pH and temperature were maintained at 5.6 and 35°C respectively and the incubation period was 40 hours. The cultivation of cells using refined olive oil as carbon source acts as inducer for the production of membrane bound lipase ROL 31 and ROL 34 [20]. The cell density obtained was 10^6 spores/L approximately. Following cultivation, the cells were immobilized by entrapment immobilization using sodium alginate as done for pure lipase. During the immobilization procedure each bead of the sodium alginate of uniform diameter and volume contained a specific amount of culture which constituted $10^3 \pm 100$ spores. This calculation simplifies the catalyst concentration as now the number of beads having a known number of cells can be taken as a measure. After immobilization, the cells were subjected to algal oil pre-treatment for 24 hours. Extracted algal oil was used for pre-treatment as this showed to improve the mass transfer rate during batch operation. The activity of the catalyst is defined as one milli equivalent of free fatty acid per minute per gram of triglyceride sample. The activity of the Whole Cell Biocatalyst was determined by the procedure explained for determination of lipase activity.

2.5. Optimization of reaction parameters for transesterification by biocatalysts

All the reactions were carried out in 50 ml screw cap flasks kept inside a temperature controlled orbital shakers. The optimization procedure was carried out for methanol as acyl acceptor and the optimum conditions were used for other six acyl acceptors. All the reaction flasks along with the control were added with suitable amounts of n-hexane (as organic solvent), 0.1 M phosphate buffer (to regulate the pH) and 3 Å molecular weight sieves (for removing water from the reaction system).

The important factors which were known to influence the yield of the transesterification by lipase and whole cell biocatalyst were optimized to obtain the highest yield. The transesterification parameters such as Temperature (20°C-40°C), Molar ratio of oil to alcohol (1:1-1:5), Catalyst concentration (5%-15% for lipase and 30-50 Beads for whole cell biocatalyst), pH (5-10), Rate of stirring (125-225 rpm) and time (0-36 hours for lipase and 0-72 hours for whole cell biocatalyst). The catalysis was done in separate flasks for each of the parameter for immobilized lipase and whole cell biocatalyst. The analysis of the biodiesel using GC-MS was done in triplets. After the optimization of

the reaction conditions, a comparative analysis of the biodiesel yield for seven different acyl acceptors was done for both the biocatalyst. Acyl acceptors including Methanol, Ethanol, Propanol, Butanol, Iso-propanol, Iso-butanol and Iso-amyl alcohol were used to find the yield of biodiesel under the previously optimized conditions.

2.6. Fatty Acid Profiling and characterization

Fatty acid composition of the algal oil and the yield of biodiesel was analysed by gas chromatography-mass spectrometry equipped with VF-5 MS capillary column (5 % phenyl-95% methyl polysilaxane) with dimensions of 30 mm * 0.25 mm* 0.25 mm. The column temperature of each run was started at 90°C for 2 min, then raised to 300°C and maintained at 300°C for 10 minutes. The alkyl esters in the catalysis procedure was analysed using a gas chromatogram equipped will CHEMITO GC 8610 flame ionization detector with BFX-70(50% cyno propyl 50 % methyl silaxane) column. Injection port was maintained at 250°C and detector port at 260°C. Oven's starting temperature is 160°C and was raised by 15°C for every two minutes. Suitable fatty acid alkyl esters were used as standard for their respective analysis.

From the GC-MS analysis, the conversion can be determined using the formula, [21]

$$Conversion\ (\%) = \frac{m_{ester}}{3\times\frac{m_{oil}}{MW_{oil}}\times MW_{ester}} \times 100 \quad (4)$$

Where the m_{ester} indicates the weight of methyl ester (g) and m_{oil} indicates the weight of oil sample (g).

The average molecular weight of algal oil (MW_{oil}) and molecular weight of fatty acid i (MW_i) can be calculated using the formula (5) and (6) with the knowledge of percentage of fatty acid ($\%m_i$).

$$MW_{oil} = 3 \times \sum_i (MW_i \times \%\ M_i) + 38 \quad (5)$$

$$MW_{ester} = \sum_i (MW_i \times \%\ M_i) + 14 \quad (6)$$

The oil properties such as Saponification value (SV), Iodine Value (IV), Degree of unsaturation (DU) and Long-Chain Saturated Factor (LCSF) can be estimated theoretically [22] using the following formulas (7), (8), (9) and (10), where D is the number of double bonds, M is the FA molecular mass, and N is the percentage of each FA component of the algal oil,

$$Saponification\ value\ (SV) = \sum(560 \times N)/M \quad (7)$$

$$Iodine\ value\ (IV) = \sum(254 \times D \times N)/M \quad (8)$$

$$Degree\ of\ Unsaturation\ (DU) = MUFA + (2 \times PUFA) \quad (9)$$

$$Long\ Chain\ Saturated\ Factor\ (LCSF) = (0.1 \times C_{16}) + (0.5 \times C_{18}) + (1 \times C_{20}) + (1.5 \times C_{22}) + (2 \times C_{24}) \quad (10)$$

The fatty acid chain length and the position of double bonds in the fatty acid alkyl esters produced by the transesterification influence the fuel characteristics of the biodiesel [23] such as Cetane number (CN), Cold Filter Plugging Point (CFPP) and Cloud point (CP). These properties can be calculated using the following formulas (11), (12) and (13),

$$Cetane\ Number\ (CN) = 46.3 + \left(\frac{5.458}{SV}\right) - (0.225 \times IV) \quad (11)$$

$$Cold\ Filter\ Plugging\ Point\ (CFPP) = (3.1417 \times LCSF) - 16.477 \quad (12)$$

$$Cloud\ Point\ (CP) = (0.526 \times C_{16}) - 4.992 \quad (13)$$

RESULTS AND DISCUSSION

3.1. Oil extraction and Biocatalyst activity

The humidity content present in the sample was found to be 7.78 ± 0.25%. It is necessary to find the humidity content of the sample and bring it to a constant minimum value as the unbound water present in the sample inhibits the penetration of the organic solvent during oil extraction by soxhlet method. Also the water that is present in the oil influences the activity of the lipase catalyst. In order to avoid the unfavourable influence of the water in the form of humidity it is necessary to reduce it to a minimum amount. The lipid obtained from the macroalgae after soxhlet extraction using hexane was 12.21% after removing the chlorophyll present in the oil by adsorption on to activated charcoal.

The catalytic activity of immobilized lipase was 31 I/mg and the catalytic activity of immobilized whole cell *Rhizopus oryzae* was 22 IU/mg.

3.2. Comparative Analysis of reaction parameters for Purified lipase and Whole Cell Biocatalysts

In order to obtain a maximum yield of biodiesel, several reaction parameters need to be optimized which includes the temperature, molar ratio of oil to alcohol, catalyst concentration, pH, rate of stirring and time. These reaction parameters were optimized for both immobilized lipase and immobilized whole cell *Rhizopus oryzae* and their comparative analysis is explained below.

3.2.1. Temperature

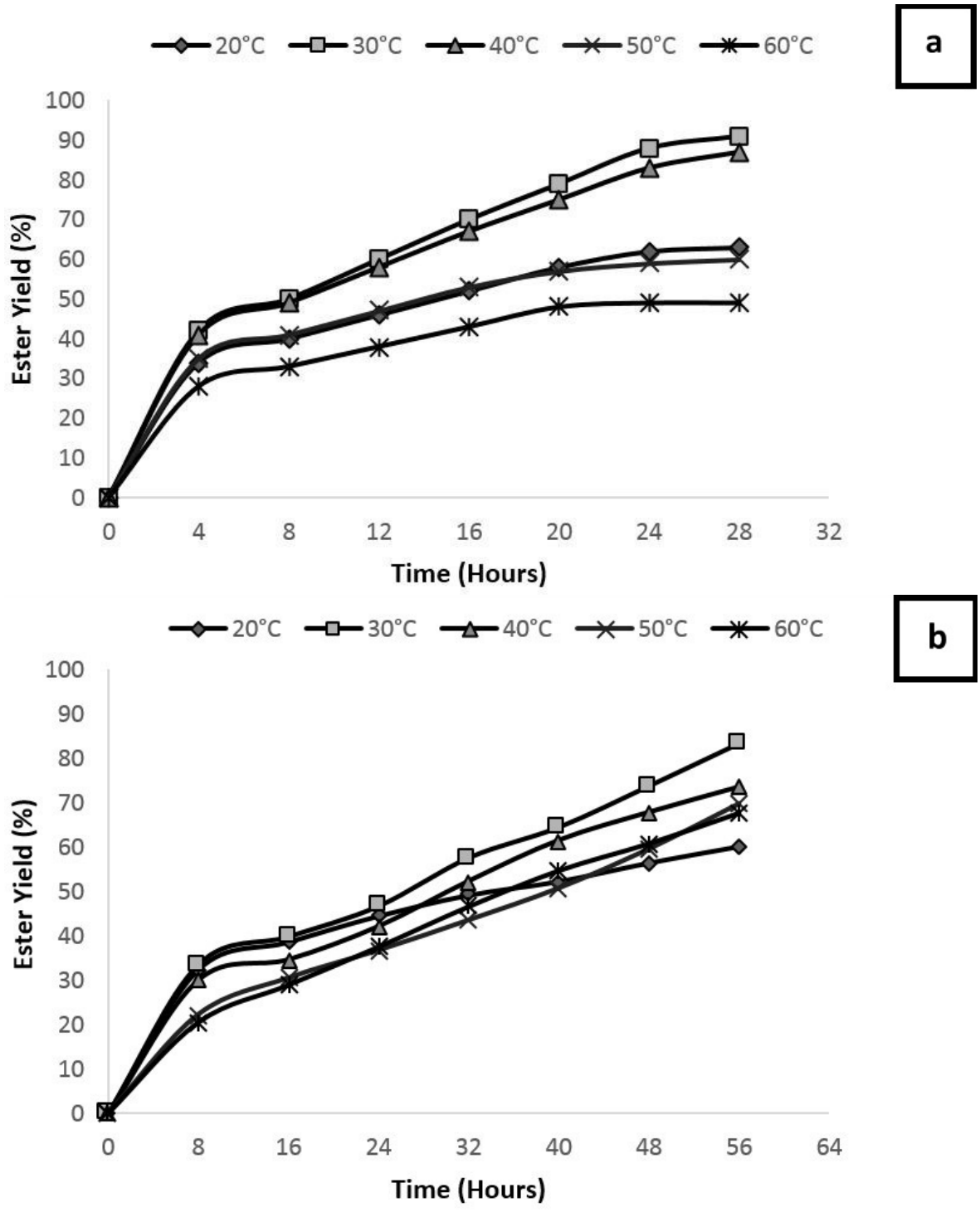
The temperature factor plays a crucial role in governing the final yield of biodiesel produced by enzyme catalysis. Fig. 1(a) depicts the optimization plot for lipase catalyzed biodiesel production from algal oil. In the case of immobilized lipase, the yield of biodiesel produced was increased from 63.6% for 20°C to 90.4% for 30°C at the end of 28 hours reaction time which remained constant thereafter. A decrease in yield was observed with increase in temperature with catalysis at 60°C giving only a yield of 49.8% at the end of 28 hours. Fig. 1(b) depicts the optimization plot for whole cell biocatalyst catalyzed biodiesel production from algal oil. For Whole cell biocatalysts, maximum yield of 83.4% was obtained for 30°C in a catalysis period of 56 hours. Further increase in catalysis temperature did not increase the final yield. The lowest yield was recorded for the temperature of 20°C unlike the highest temperature of 60°C, which was 60.2%. Raise in temperature for catalysis using whole cell biocatalyst showed reduction in yield of biodiesel in a manner as similar to that of the immobilized lipase but the stability of the catalyst was more for whole cell rather than lipase as whole cell catalyst gave a comparatively higher yield than lipase at high temperature.

Increasing reaction temperature moves the reaction rate in the forward direction and enables the attainment of equilibrium at a shorter time period. Also, increase in temperature reduces the viscosity of the algal oil thereby enabling the solubility of the oil in the acyl acceptor. This also helps in improving the net yield of biodiesel as diffusional problems are eliminated. Another factor that is influenced by the change in temperature is the stability of the biocatalyst. Lipase is stable only up to a temperature of 55°C, beyond that temperature the enzyme denatures. Also, the spores of fungi *Rhizopus oryzae* are killed at high temperatures of above 75°C. Retention of catalytic activity by the biocatalyst after the catalysis was found to be possible only in the temperature range of 30-40°C. A temperature of 30°C was found to be optimum for transesterification by lipase and whole cell biocatalyst.

3.2.2. Molar ratio of oil to alcohol

Acyl acceptor (Alcohol) is an important reactant in the transesterification of oil. Amount of this alcohol is an important factor governing the final biodiesel yield. Fig. 2(a). Shows the influence of the amount of alcohol on the yield of biodiesel for lipase catalysis. Highest conversion rate of 89.2% was achieved for oil amount of 1:3 to that of alcohol amount (v/v). The lowest conversion was found at the extremes of oil amount to alcohol amount (that is 1:1 and 1:5), which was 52.8% and 64.3% respectively. Fig. 2(b) shows the influence of the amount of alcohol on the yield of biodiesel for whole cell catalyst. Highest yield of 85.5% was obtained for oil to alcohol ratio of 1:3. An interesting result was obtained in the case of whole cell biocatalyst with varied ratios of alcohol, the final biodiesel yield for oil to alcohol ratio of 1:1, 1:2, 1:4 and 1:5 was 71.6%, 71.7%, 71.6% and 68.5% respectively.

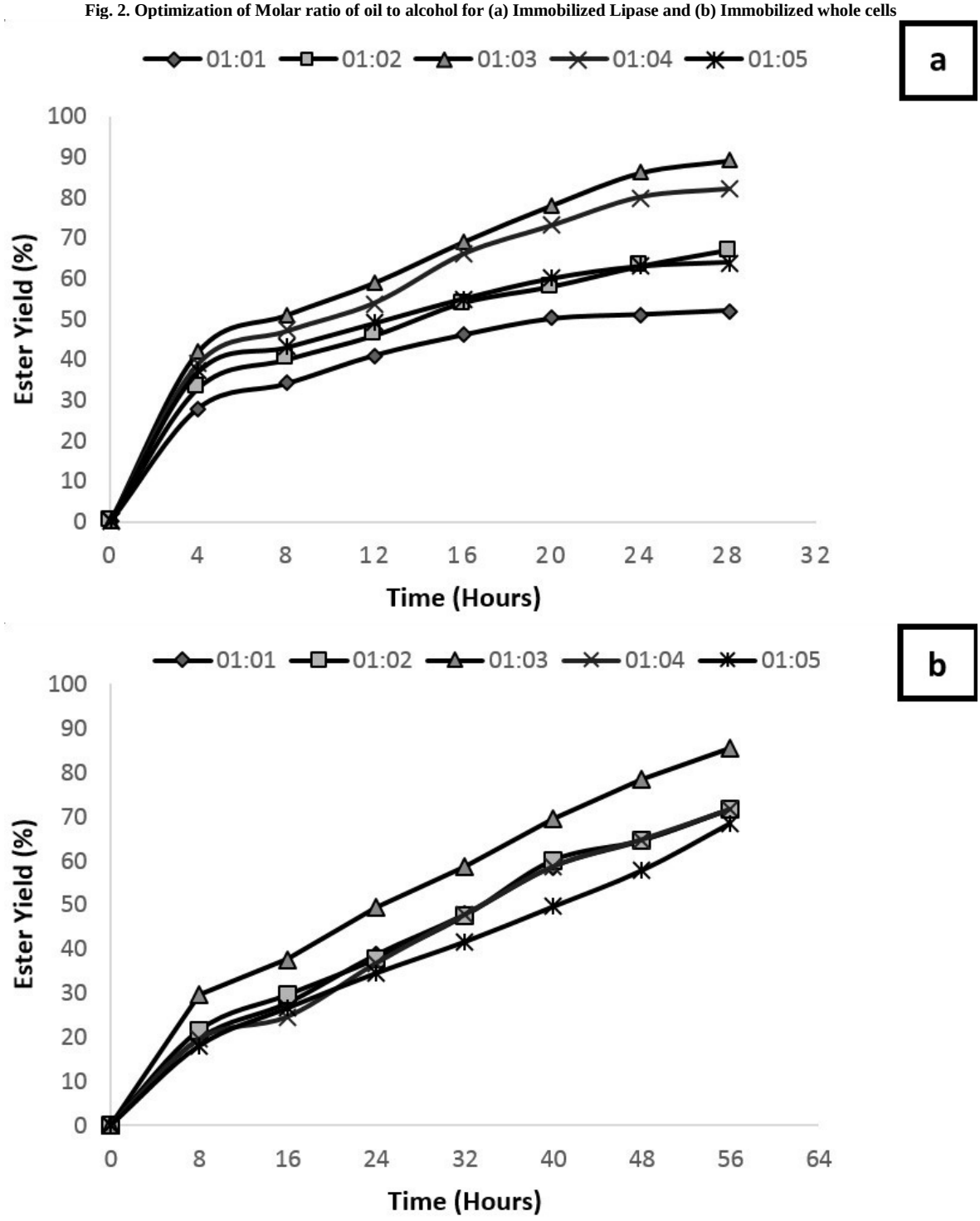
Fig. 1. Optimization of Temperature for (a) Immobilized Lipase and (b) Immobilized whole cells



This result proved that the activity of the lipase inside cell remains stable at higher amounts of alcohol as compared with purified lipase hence showing greater stability against methanol denaturation. Influence of the amount of alcohol was seen in driving the reaction equilibrium in the forward direction and also in denaturation of the catalyst. Amounts of alcohol theoretically required for producing esters from oil is three moles. Any amount more than that

theoretical amount proved to drive the reaction in to ester formation at a faster rate and maintain it at that state without reverse reaction. But a higher amount of alcohol proved to denature the catalyst. Hence the optimum amount is necessary. Lipase was easily denatured with prolonged exposure to higher alcohol amount of alcohol whereas whole cell biocatalyst showed stability. A molar ratio of 1:3 for oil to alcohol was found to be optimum for transesterification by lipase and whole cell biocatalyst.

Fig. 2. Optimization of Molar ratio of oil to alcohol for (a) Immobilized Lipase and (b) Immobilized whole cells



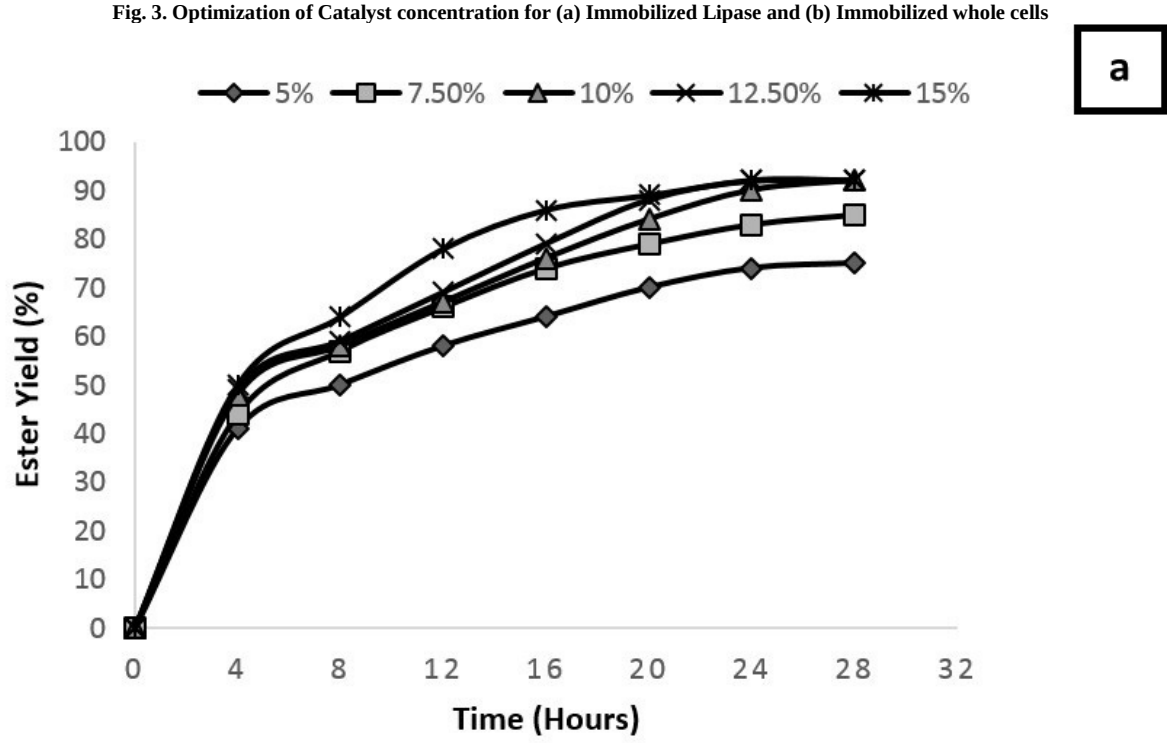
3.2.3. Catalyst concentration

The effect of purified lipase concentration on biodiesel yield (as shown in Fig. 3(a).) was studied with different catalyst concentrations of 5, 7.5, 10, 12.5 and 15% (wt. %). The other initial parameters were 3:1 methanol to algal oil molar ratio, 175 rpm of mixing intensity, 30 °C of reaction temperature for 28 hours of reaction time. As the Lipase concentration increased, biodiesel yield was found to be increased. Insufficient amount of catalyst resulted in incomplete conversion of triglycerides into the biodiesel as indicated from its lower yield of 75.7%. The ME yield was increased from 75.7% to 92.4% with increase in the catalyst concentration from 5% to 15%. The maximum biodiesel yield of 92% was achieved at 28 hours, when the lipase concentration was 10%. Further increase in lipase concentration to 15% did not increase the yield and the same biodiesel yield of 92% was obtained.

The effect of whole cell biocatalyst concentration on biodiesel yield (as shown in Fig. 3(b).) was studied with different number of immobilized whole cell beads with a constant number of cells 30, 35, 40, 45 and 50. Unlike lipase immobilization on weight proportion, for whole cell biocatalysts the number of biomass supporting particle (immobilized bead) is considered. Approximately $10^3 \pm 100$ cells will be present in each bead. Like immobilized lipase, for whole cell biocatalyst a similar pattern of increase in biodiesel yield with catalyst amount was observed. Maximum conversion of 81.6% was observed for 50 beads, beyond which there was no significant change. The transesterification process is controlled by the immobilized biocatalyst lipase and whole cells of *Rhizopus oryzae*.

The concentration of the catalyst determines the active sites available for the reactants to form products. Hence the concentration of the catalyst above a minimum limit should be available in order to obtain the maximum biodiesel yield in the minimum duration of time. Another contributing factor towards the biodiesel yield is the mass transfer inhibitions in the case of immobilized catalysts. Optimum amount of biocatalyst should be present to avoid the overcrowding and mass transfer problems as excess of biocatalyst is not desirable. A catalyst concentration of 12.5% was optimum for lipase catalyzed transesterification and 50 beads for whole cell catalyzed transesterification.

Fig. 3. Optimization of Catalyst concentration for (a) Immobilized Lipase and (b) Immobilized whole cells



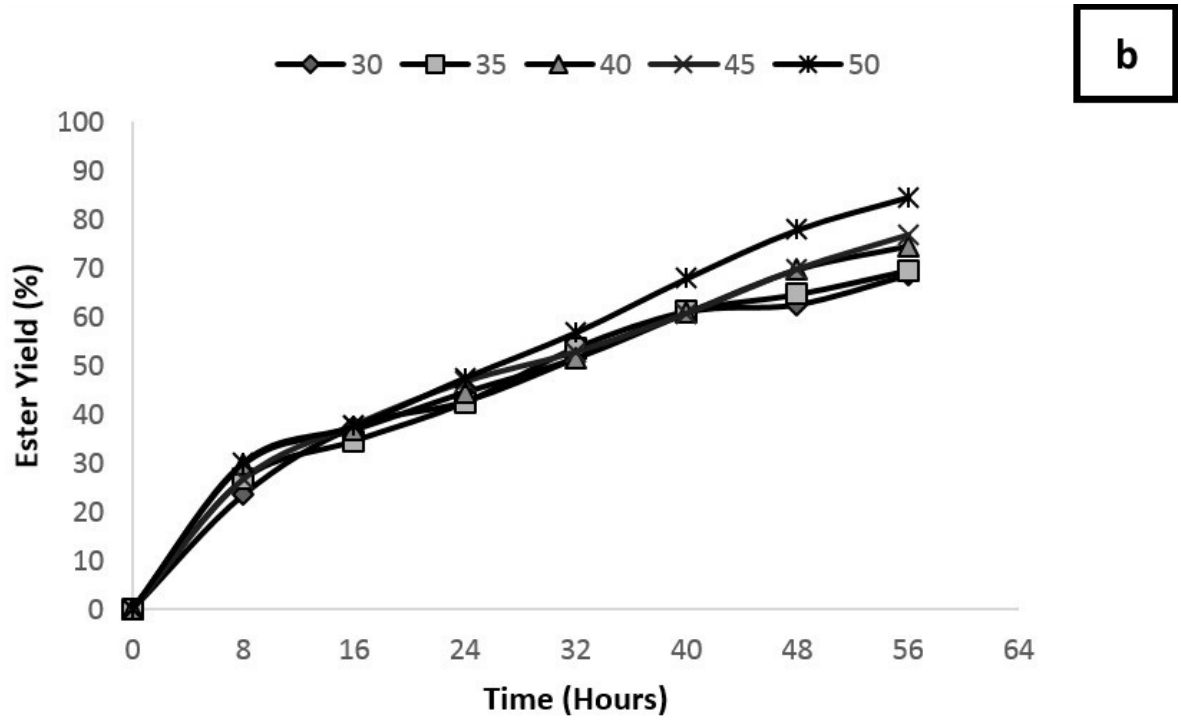
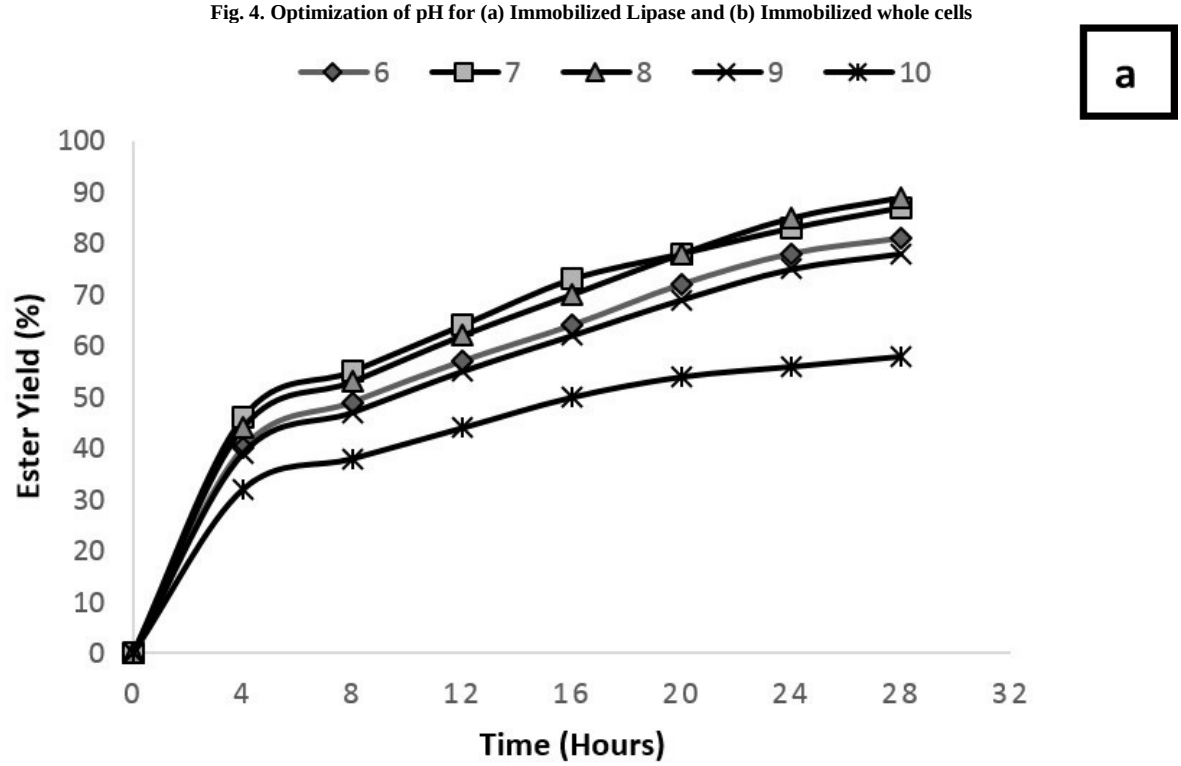
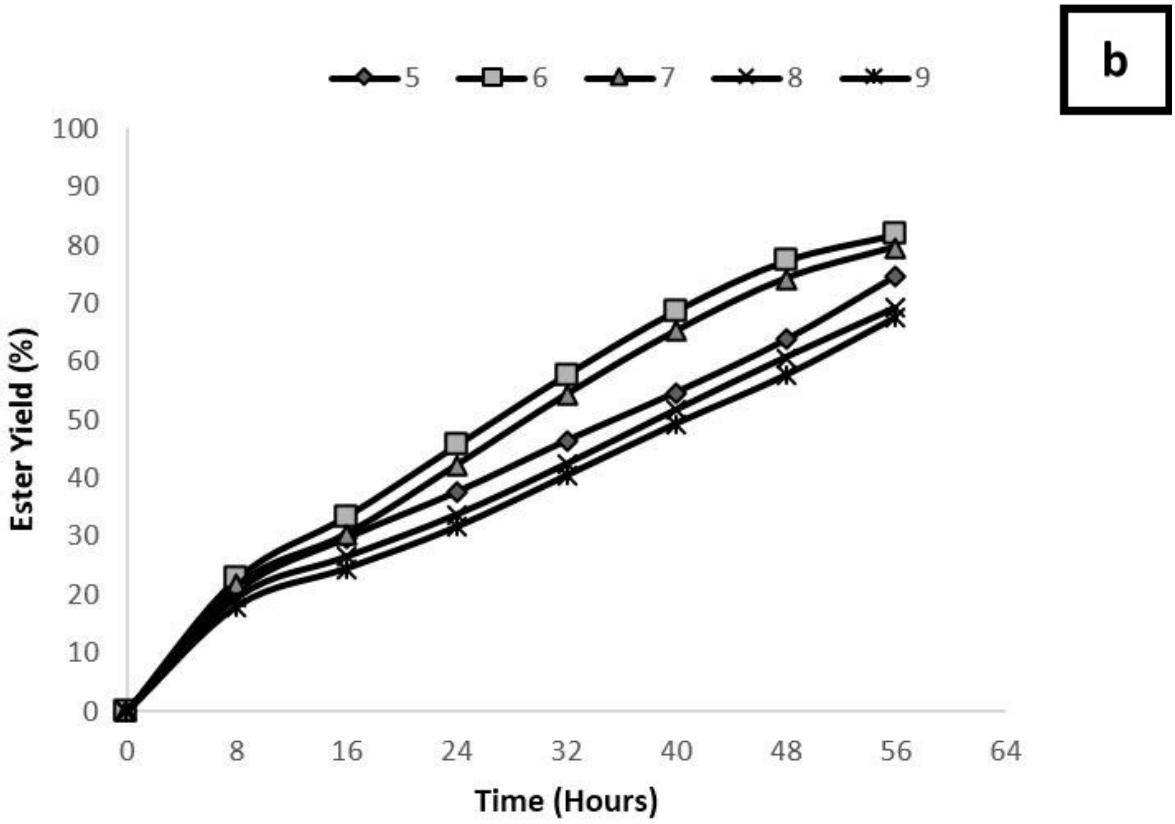


Fig. 4. Optimization of pH for (a) Immobilized Lipase and (b) Immobilized whole cells





3.2.4. pH

Influence of pH on lipase catalysis in the Fig. 4(a)., it can be seen that the maximum yield at conditions of 30°C, 1:3 molar ratio of oil to alcohol, 10% catalyst concentration and 150 rpm was obtained for pH 7 as 89.7% after 28 hours of reaction time. Alkali conditions of pH higher than 9 seriously damaged the biodiesel yield to 78.4% and 58.9% for pH 10. Acidic pH above 5.5 did not have any serious effect on the net yield but increased the time period for obtaining the desired yield. Influence of pH on whole cell catalysis in the Fig. 4(b), it can be seen that the yield of biodiesel decreases with the increase in pH. Optimum pH is pH 6 and it gives a yield of 81.8%. The lowest yield was obtained for pH 9 as 67.5%. The decrease in the yield was not as drastic as that of purified lipase for whole cells, as whole cells showed more stability to changes in microenvironment than lipase.

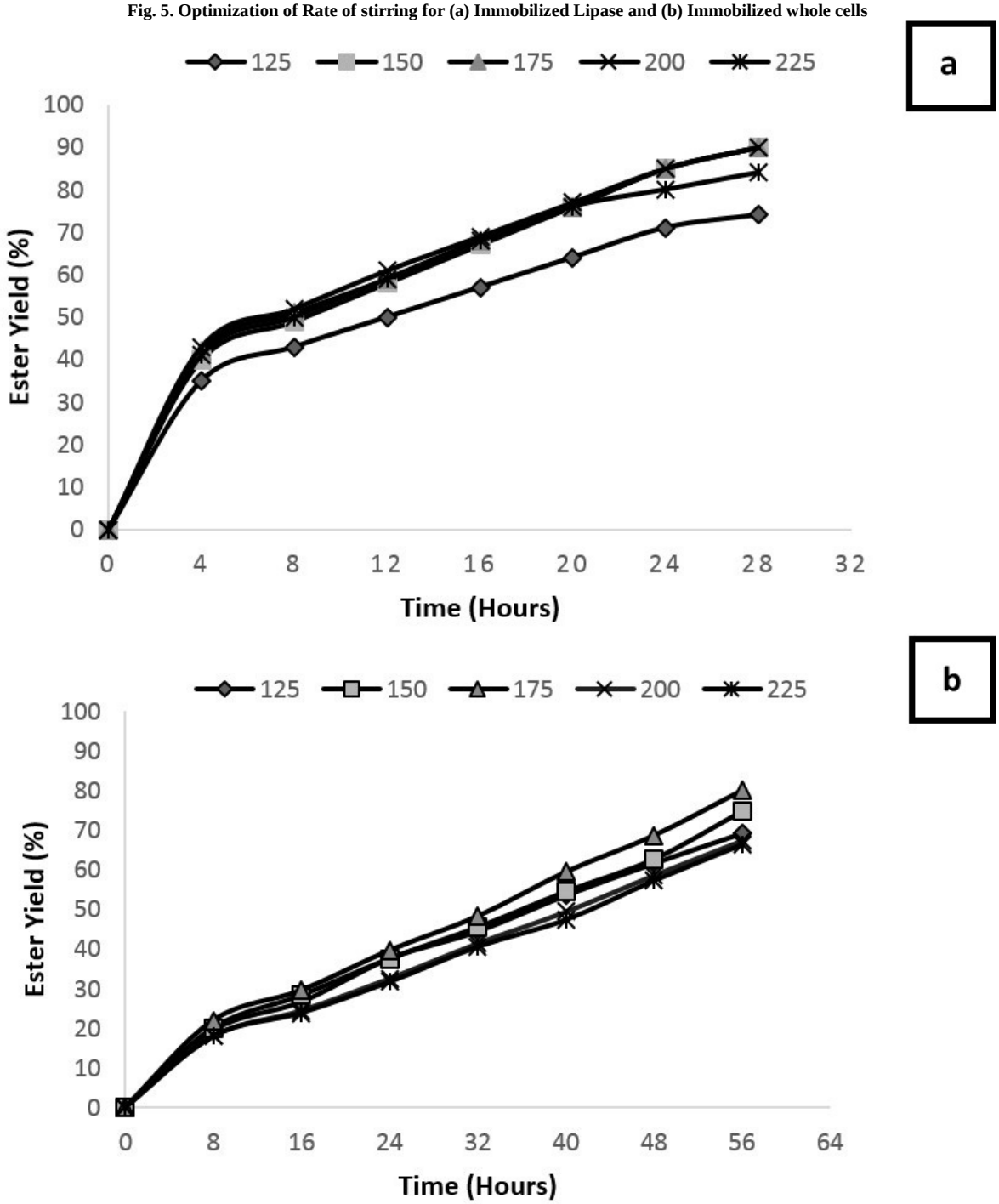
Electrostatic charges of polar groups contained in enzyme molecules depend on pH of their microenvironment and affect the rate of transesterification. Adjustment of the pH of the reaction mixture before the start of the catalysis enables in the retention of the enzyme activity. Sudden changes in the pH denatures the lipase and may cause changes in the activity and consequently lower the biodiesel yield. The pH of the reaction mixture does not have any direct influence on the biodiesel yield but it indirectly affects the enzyme activity. This can be said true even for the whole cell biocatalysts as the changes in the environment around the cell causes the changes in the metabolism and the target enzyme production can be hampered. A pH of 7 was optimum for lipase catalyzed transesterification and pH 6 for whole cell catalyzed transesterification.

3.2.5. Rate of stirring

Agitation or stirring is necessary to initiate the process of transesterification by mixing the bi-phasic reactants (alcohol and oil). It is an important parameter during scaling up of the transesterification procedure. Fig. 5(a) shows the observation for the yield of biodiesel under different rates of stirring for lipase catalyzed transesterification. Fig. 5(b) shows the observation for yield of biodiesel under different rates of stirring for whole cell catalyzed transesterification. The maximum yield of 90.4% and 80.1% was obtained at 175 rpm for lipase and whole cell respectively. At low stirring rate of 125 rpm the yield obtained after the reaction time was 69.3% and 74.3%

respectively for whole cell and lipase. The possible reason for this lower yield is that improper mixing has reduced the chance of exposure of the reactants to the catalyst thereby lowering the reaction rate. High rate of stirring has shown to also reduce the yield. Rate of stirring at 225 rpm has reduced the yield to 80.6% and 66.4% for lipase and whole cell respectively.

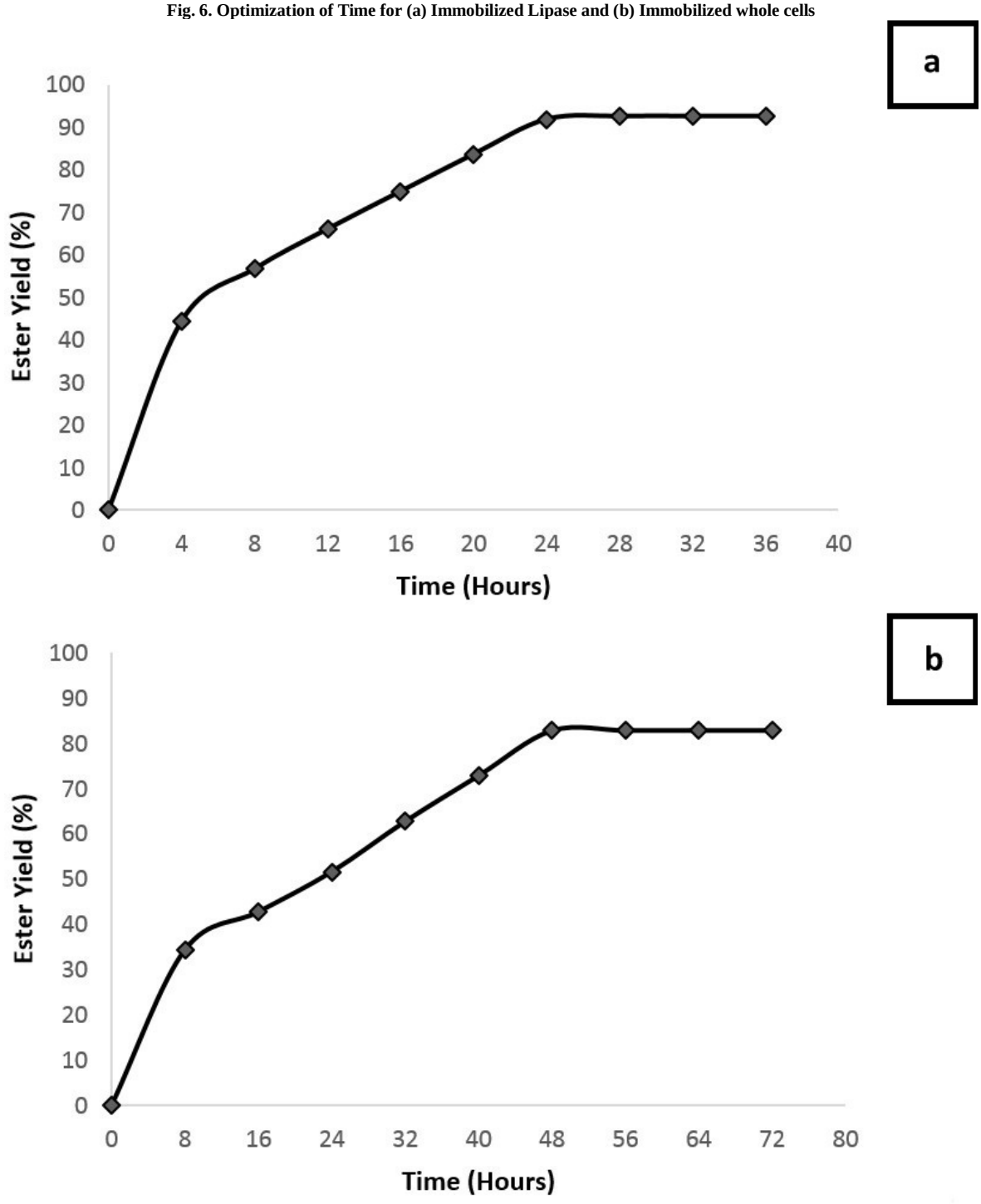
Fig. 5. Optimization of Rate of stirring for (a) Immobilized Lipase and (b) Immobilized whole cells



Diffusion of reactants and products through the pores of the immobilized catalyst is governed by the rate of stirring. Glycerol (another product of transesterification) and methanol are denaturing agents. They need to be quickly

removed from the surface of the catalyst to improve the reaction rate and yield. In order to facilitate regulated removal of denaturants and introduction of the reactants to the catalyst the rate of stirring is being optimized. A rate of stirring of 150 rpm was optimum for lipase catalyzed transesterification and 175 rpm for whole cell catalyzed transesterification.

Fig. 6. Optimization of Time for (a) Immobilized Lipase and (b) Immobilized whole cells



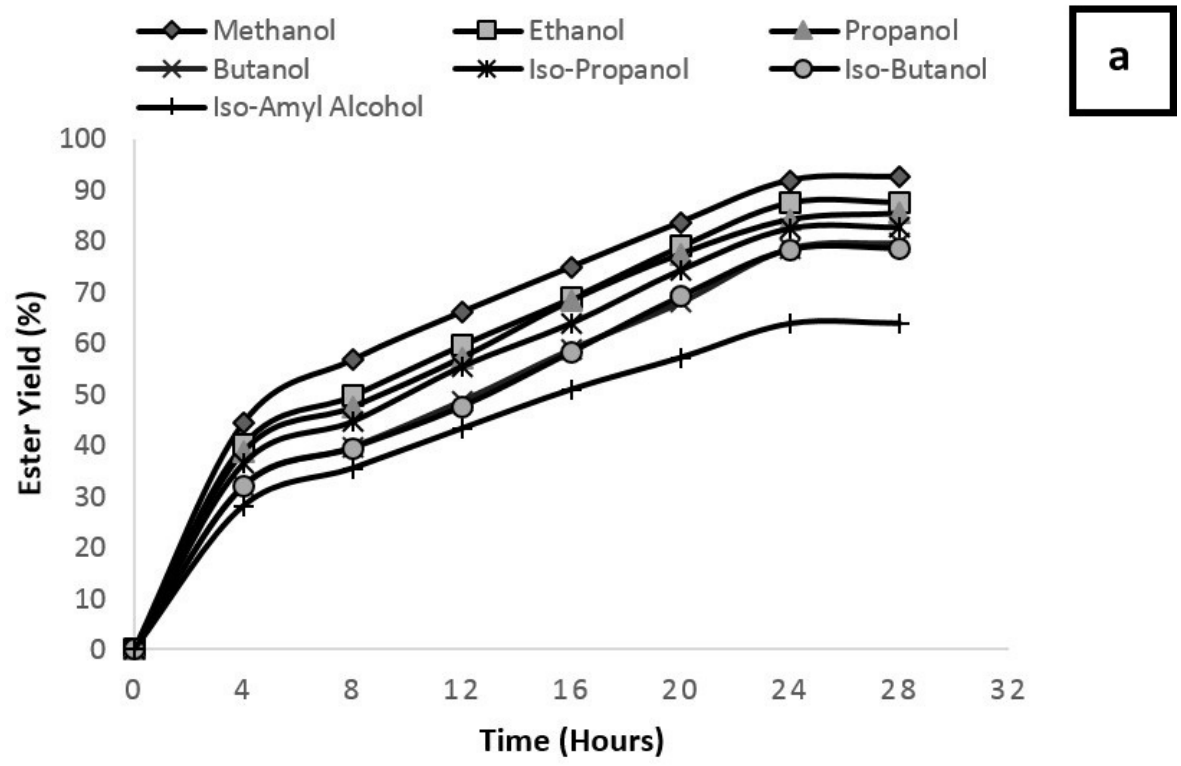
3.2.6. Time

Reaction time is a crucial parameter in deciding the feasibility of any production process. The time taken for any process to attain its equilibrium should not be long. If the time taken for a reaction to attain equilibrium is more then it means that the reaction is not feasible or requires the use of external energy or catalyst. Time period of a catalytic process is therefore very important. In the Fig. 6(a) the reaction time for lipase catalysis is shown. The yield was examined for every 4 hours for 36 hours at conditions of 30°C, 12.5 % catalyst concentration, 1:3 oil to alcohol ratio, 150 rpm, 7 pH and methanol as acyl acceptor. Maximum yield of 91.8% for lipase was obtained at the end of 24 hours, beyond which there was no significant change in the yield. In the Fig. 6(b) the reaction time for whole cell catalysis is shown. The yield was examined for every 8 hours for 72 hours at conditions of 30°C, 50 beads, 1:3 oil to alcohol ratio, 175 rpm, 6 pH and methanol as acyl acceptor. Maximum yield of 82.8% for lipase was obtained at the end of 48 hours, beyond which there was no significant change in the yield. A time period of 24 hours was optimum for lipase catalyzed transesterification and 48 hours for whole cell catalyzed transesterification.

3.2.7. Acyl acceptors

Acyl acceptor is the other reactant apart from the oil in a transesterification reaction which exchanges an alkyl chain with a triglyceride to form the ester product. Alcohols as acyl acceptors based on their ability to move the reaction in the forward direction, chemical structure, availability and denaturing potential have been selected to determine their ability to produce with biocatalysts. Seven alcohols namely Methanol, Ethanol, Propanol, Butanol, Iso-propanol, Iso-butanol and Iso-Amyl alcohol was analysed for their potential to yield biodiesel.

Fig. 7. Optimization of Acyl acceptors for (a) Immobilized Lipase and (b) Immobilized whole cells



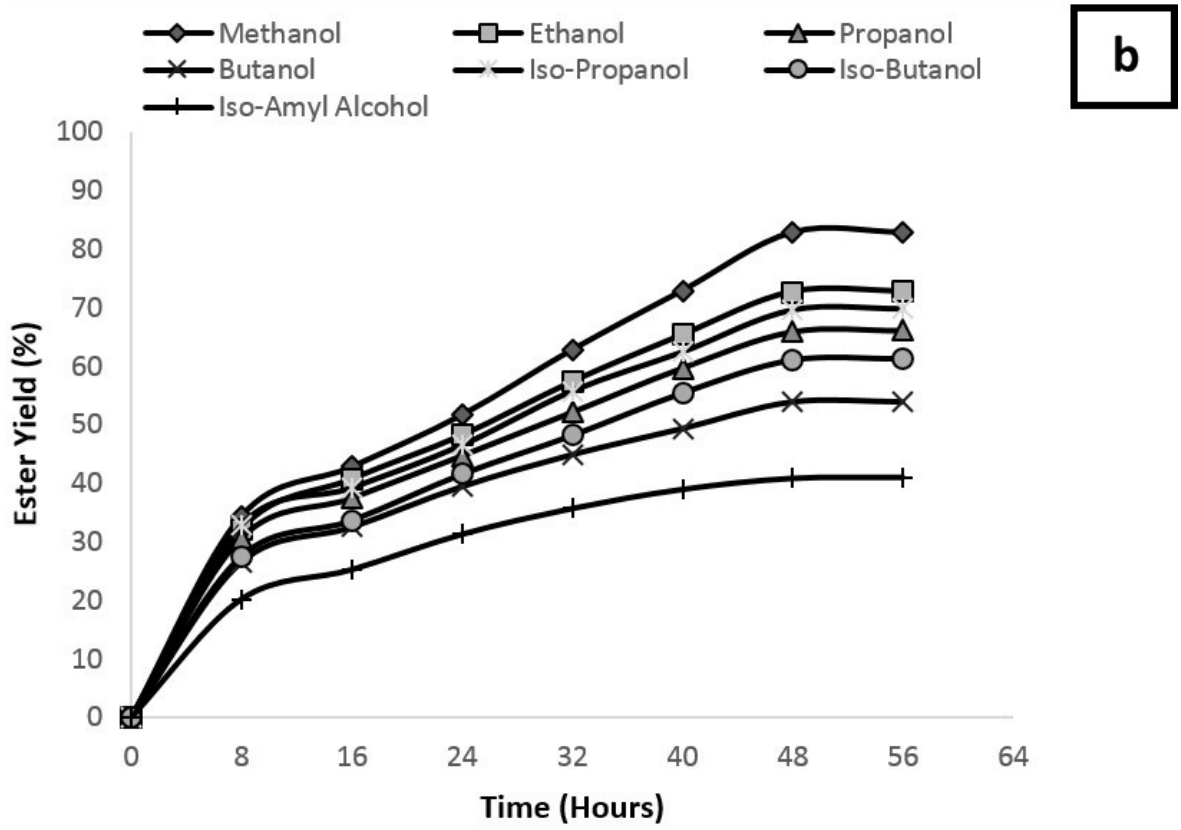


Fig. 7(a) Illustrates the lipase catalyzed yield of biodiesel for seven different acyl acceptors in the ratio of 3:1 with oil at optimized conditions of temperature (30°C), pH (7), Reaction Time (24h), Rate of stirring (150 rpm) and enzyme concentration (12.5%). Maximum biodiesel yield of 92.5% was obtained for methanol, followed by 87.6%, 85.4%, 79.6%, 82.7%, 78.5% and 63.8% for Ethanol, Propanol, Butanol, Iso-propanol, Iso-butanol and Iso-Amyl alcohol respectively at the end of 24 hours. Similarly for whole cell biocatalyst, Fig. 7(b) Illustrates the biodiesel yield at optimized conditions of temperature (30°C), pH (6), Reaction Time (56h), Rate of stirring (175 rpm) and enzyme concentration (50 beads). The yield for the seven acyl acceptors are 82.8%, 72.7%, 66%, 53.8%, 69.7%, 61.2% and 40.9% respectively at the end of 56 hours of reaction time. Maximum yield was obtained for methanol for both the biocatalyst followed by relatively appreciable yield for ethanol. Lowest yield was obtained for Iso-Amyl alcohol for both biocatalyst which is due to its high molecular weight and complex structure which reduced its miscibility and diffusion.

The variations in the yield of biodiesel with different alcohols can be attributed to the differences in the structure and molecular weight of the alcohol which influence the diffusion and the rate of catalysis. Methanol and ethanol have the smallest molecular weight hence can diffuse easily, but both are strong denaturants. The C3-C5 alcohols have higher molecular weight which restricts their diffusion, but are not stronger denaturants. As it is known that alcohol is a strong denaturant of the biocatalyst it is necessary to choose the alcohol wisely considering the fact that yield of transesterification by biocatalyst is an interplay between rate of enzyme denaturation and enzyme catalysis.

3.3. Fatty acid profile and oil characteristics

The fatty acid analysis of the *Enteromorpha compressa* oil gave the following compositional data which is illustrated in the Table 1. High amounts of C16 fatty acids (44.64%) and C18 fatty acids (33.14%) were present in the oil obtained. The total saturated fatty acids (SFA) present in the oil was 47.60%, total mono unsaturated fatty acids (MUFA) present in the oil was 7.94% and total poly unsaturated fatty acids (PUFA) present in the oil was 28.33%.

Table 1 Fatty acid composition of *Enteromorpha compressa* oil

Fatty Acid Type	Composition (Wt %)	Molecular Weight (g/mol)
12:0	0.10 ± 0.01	200.32
14:0	0.91 ± 0.05	228.37
16:0	44.64 ± 1.34	256.43
17:0	0.33 ± 0.02	270.46
18:0	0.75 ± 0.04	284.48
20:0	0.35 ± 0.02	312.54
22:0	0.52 ± 0.03	340.59
16:1n-7	0.80 ± 0.04	254.43
18:1n-9	1.31 ± 0.07	282.48
18:1n-7	5.50 ± 0.17	282.48
20:1n-9	0.12 ± 0.01	310.51
22:1	0.21 ± 0.01	338.59
18:2n-6	5.79 ± 0.29	280.45
18:3n-6	0.85 ± 0.04	278.43
20:2n-6	0.23 ± 0.01	308.49
20:3n-6	0.20 ± 0.01	306.48
20:4n-6	0.49 ± 0.02	304.47
18:3n-3	14.08 ± 0.70	278.43
18:4n-3	4.86 ± 0.10	276.40
20:3n-3	0.10 ± 0.01	306.45
20:5n-3	0.54 ± 0.03	302.45
22:5n-3	1.190 ± 0.06	330.59

The characteristics of the oil and the biodiesel which are required to qualify them to be used as a fuel source were analysed and the results showed that the saponification value is 152.99, Iodine value is 99.57, Degree of unsaturation is 64.6% and and Long-Chain Saturated Factor is 5.969. The biodiesel properties such as Cetane number, Cold Filter Plugging Point and Cloud point were determined to be 59.57, 2.27 and 18.48 °C respectively. These calculations are based on the fatty acid composition of the oil and are not done based on engine testing in accordance with international standards. These results theoretically prove that the algal oil and its subsequent ester have the potential to be used as fuel.

CONCLUSION

The usage of *Enteromorpha compressa* oil as a source for producing biodiesel catalyzed by biocatalysts immobilized lipase and immobilized *Rhizopus oryzae* cells gave good yields of 92% and 83% respectively. Further the optimization of the reaction parameters for both the catalysts showed that the biocatalysts are a good option for industrial level biodiesel production from algal oil. Results further implicated the fact that the commercial level biodiesel production can be done by utilizing the macro algal oil. The oil from macro algae will considerably reduce the dependence on terrestrial oil crops for energy generation. The major challenge in the utilization of macro algal oil is the disposal of the large amount of biomass which is accumulated after the oil extraction process. Suitable methods or processes which utilize this spent biomass for producing useful products can be integrated with the biodiesel production process.

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