Enzymatic transesterification of rubber seed oil using Rhizopus Oryzae Lipase

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Abstract

Biodiesel production from non-edible rubber seed oil with high free fatty acid (FFA 26\%) content was investigated. Rhizopus Oryzae Lipase in the form of free powder was used as catalyst. The effect of reaction parameters such as catalyst concentration, reaction duration, pH of the reaction mixture, type of acyl acceptors used and oil to acyl acceptor molar ratio were studied. Ethyl acetate gave better results in comparison with methanol as acyl acceptor. A maximum conversion of 33.3\% was obtained when ethyl acetate was used, at a molar ratio of 1:11 and catalyst concentration 10 (w/w) \% of oil after 24 hours. In case of methanol, maximum conversion was 31\% at a molar ratio of 1:4 and catalyst concentration of 15(w/w) \% of oil after 48 hours. Variation in pH did not show any significant change in percentage of conversion.

Keywords: Biodiesel; Rhizopus Oryzae Lipase; Rubber seed oil; Transesterification

1. Introduction

Diminishing fossil fuel reserves, environmental hazards associated with fossil fuel combustion emissions and increasing energy needs are the main reasons for researches in alternative fuels. Biodiesel is an attractive alternative fuel because of its ease in production and replacement potential of diesel. Biodiesel can be produced from edible oils (e.g.: coconut oil, sunflower oil etc.) as well as non-edible oils (e.g.: rubber seed oil, cotton seed oil etc.). In a country like India where food security is yet to be achieved, use of edible oils for biodiesel production will arise a lot

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of moral queries. So it is necessary to use non-edible oils for biodiesel production. The estimated availability of rubber seeds in India is 30,000 tons per annum, from which approximately 5000 tons of rubber seed oil can be produced [1]. Nowadays rubber seed oil is only used in soap and paint industries. That means naturally produced rubber seed itself is heavily underutilized. So rubber seed oil can be considered as a potential feed stock for biodiesel production.

Blending, emulsification, thermal cracking and transesterification are the commonly adoptable methods to use the vegetable oil as fuel in diesel engines [2]. Among these transesterification is most popular method. In the transesterification of vegetable oils, a triglyceride reacts with an alcohol in the presence of catalyst, producing a mixture of fatty acids alkyl esters and glycerol [3]. The catalyst can be a strong acid or base for chemical transesterification and it can be a lipase in case of enzymatic transesterification. The chemical transesterification of vegetable oils to biodiesel has been industrially adopted due to its high conversion rates and lower production time. Chemical catalysts used for industrial biodiesel production (usually NaOH, KOH or sodium methoxide) provide the yield of transesterification reaction close to 99% [4]. However, this process has several drawbacks such as energy-demanding and environmentally unfriendly processing steps (e.g.: catalyst and product recovery, and waste water treatment). If the FFA of oil used is above 0.5% saponification will happen and hence reduction in biodiesel yield [5]. To overcome this issue, acid esterification has to be done. These kinds of multistage processing steps will add up to the total production cost. The cost of biodiesel currently is approximately 30% higher than that of petroleum-based diesel [6]. 60 to 80% of these costs are associated with cost of the feedstock oil used. In case of chemical transesterification the oil has to be pretreated (e.g.: degumming, dehydration etc.) which further increases the production cost. These drawbacks led to search of new catalysts, and use of enzymes will be the solution.

Enzymes are biological molecules which act as catalyst. A few of them have the capability to catalyse hydrolysis of fatty acids which are known as lipases. A lot of researches have been already done in enzymatic transesterification, and most of the results show that enzymes are good alternatives for chemical catalysts. Jilse et al. [7] has used Candida Antartica lipase B as catalyst and obtained 85% conversion on rubber seed oil having 41% FFA content. For the production of biodiesel from the same rubber seed oil by chemical transesterification two stage acid esterification and one stage base esterification have been employed [8]. The main advantages of enzymatic transesterification are [6,9]:

- Feed stocks with high FFA content can be used (up to 100%).
- Elimination of post process treatment costs associated with recovery of chemical catalysts.
- Oil can be used without any pretreatments (like refining, degumming etc.).
- Transesterification in single step process.

Most commonly used enzymes for transesterifications are Rhizopus Oryzae Lipase (ROL), Candida Antartica Lipase B, Steapsin etc. Results of enzymatic transesterification of a few vegetable oils reviewed from literatures are shown in Table 1.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>Yield</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soyabean oil</td>
<td>Novozym 435</td>
<td>97</td>
<td>[10]</td>
</tr>
<tr>
<td>Plant oil</td>
<td>ROL</td>
<td>90</td>
<td>[11]</td>
</tr>
<tr>
<td>Soyabean oil</td>
<td>Candida Rugosa</td>
<td>80</td>
<td>[11]</td>
</tr>
<tr>
<td>Crude palm oil</td>
<td>Lipozyme RM IM</td>
<td>12</td>
<td>[12]</td>
</tr>
<tr>
<td>Rubber seed oil</td>
<td>Steapsin</td>
<td>39</td>
<td>[13]</td>
</tr>
</tbody>
</table>
Rhizopus Oryzae lipase is a fungus which is present in dead organic matter. Lipase from Rhizopus Oryzae is capable of catalyzing transesterification reaction. Kaieda et al. [14] used ROL for the methanolysis of plant oil and a maximum yield of 90% was obtained within 48 hours. In 2014 Arumugam et al. [15] produced biodiesel from Calophyllum inophyllum oil using lipase produced from Rhizopus Oryzae. Molar ratio of methanol-to-oil 12:1, water content 15%v/v, cell concentration 20% and temperature 35 °C were found to be the optimum. Recently some works have been reported in enzymatic transesterification of rubber seed oil also. In 2010 Abdul Shukoor [13] used steapsin for enzymatic transesterification of rubber seed oil. Maximum conversion obtained was 39% when methanol was used as acyl acceptor.

The major drawback associated enzymatic transesterification is the high cost of enzyme. Slow conversion rate, loss of activity during process, enzyme activity inhibition by reactants and product are some other challenges in enzymatic transesterification. Researches are going on in the field of enzymatic transesterification so that the mentioned draw backs can be avoided. Since enzymes are not sensitive to FFA contents in raw oil, this method is a promising one to replace the lengthy and complicated chemical transesterification process. In the present study various factors affecting enzymatic transesterification of rubber seed oil using Rhizopus Oryzae Lipase were investigated.

2. Materials and methods

2.1. Materials

Rubber seed oil was purchased from Virudhnagar, Tamilnadu. Its FFA content was found as 26% by following standard AOCS titration method. Catalyst Rhizopus Oryzae Lipase (a sample pack of 50 g) was received from Biolaxi Private Limited (Bhiwandi) as a free sample. Methanol, Ethyl acetate, Sodium dihydrogen phosphate and Disodium hydrogen phosphate were purchased from Chemind Chemicals, Kozhikode.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid composition (%)</td>
<td></td>
</tr>
<tr>
<td>Palmitic acid C_{16:0}</td>
<td>10.2</td>
</tr>
<tr>
<td>Stearic acid C_{18:0}</td>
<td>8.7</td>
</tr>
<tr>
<td>Oleic acid C_{18:1}</td>
<td>24.6</td>
</tr>
<tr>
<td>Linoleic acid C_{18:2}</td>
<td>39.6</td>
</tr>
<tr>
<td>Linolenic acid C_{18:3}</td>
<td>16.3</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>0.91</td>
</tr>
<tr>
<td>Viscosity (mm²/s) at 30 °C</td>
<td>47.1</td>
</tr>
<tr>
<td>Flash point (°C)</td>
<td>210</td>
</tr>
<tr>
<td>Acid value (mgKOH/g)</td>
<td>52</td>
</tr>
</tbody>
</table>

2.2. Experimental procedure with methanol

10 ml of oil and methanol was mixed in an Erlenmeyer flask. Molar ratio followed was 1:4 and 1:5. Three step addition (535 μl in each step for 1:4) was employed to avoid chance of inhibition of enzyme activity. Measured quantity of enzyme was added to the mixture and placed in the reciprocating shaker. Enzyme quantity was expressed as percentage weight of oil (i.e. 10ml oil has a weight of 9.1g, so 1 (w/w) % of oil is 0.091g). Shaking frequency given was 170 rpm and temperature was fixed at 37 °C. After completion of the reaction, the mixture was filtered to remove the enzyme. Centrifugation was done at 5000 rpm to separate glycerol and traces of enzyme powder from
produced biodiesel. The mixture was water washed twice to remove the unreacted methanol and other impurities. Then the mixture was heated to 110 °C to remove the water content. After the completion of reaction viscosity of the samples were checked and based on the viscosity index the conversion percentage was calculated. The conversion percentage of final samples were verified by Gas Chromatography.

2.3. Experimental procedure with ethyl acetate

10 ml of oil and ethyl acetate was mixed in the Erlenmeyer flask. Molar ratios followed were 1:4, 1:7, 1:9 and 1:11 (1:1 molar ratio means for 1ml of oil 0.098 ml of ethyl acetate). Single step addition was used as ethyl acetate has no inhibition effect on enzymes. Measured quantity of enzyme was added to the mixture and placed in the reciprocating shaker. Rest of the procedure was same as that of methanol as acyl acceptor.

2.4. Important parameters

Experiments were conducted on purchased rubber seed oil with reference to the following influencing parameters on reaction:

- Catalyst concentration
- Molar ratio
- Type of acyl acceptor
- pH of the reaction mixture

During the experiments, one of the parameters varied keeping all other parameters constant to obtain the optimum value of each parameter.

3. Results and discussion

3.1. Effect of enzyme concentration

The effect of amount of Rhizopus Oryzae lipase added to the reaction mixture is shown in Fig.1. As expected the increase in catalyst concentration increased the conversion percentage. When enzyme quantity was increased from 5% to 10%, the percentage of conversion increased drastically. But above 10% there was only a small increase in conversion. So the rest of the experiments was done with 10(w/w) % of ROL considering it as optimum value. Tamalampudi et al. [16] got maximum conversion with Rhizopus Oryzae Lipase at 6 (w/w) % for Jatropha oil.

![Graph of enzyme concentration vs conversion percentage](image1)

Fig. 1. (a) Percentage of conversion with enzyme quantity; (b) Percentage of conversion with time for different catalyst concentration
3.2. Reaction rate

Samples were collected from the reaction mixture after 8, 16, 30, 48 hours respectively to know the reaction progress. The effect of time on reaction is shown in Fig. 2. For enzyme concentrations 10% and 15%, the rate was very close throughout the reaction process. Reaction mixture with 5% had shown a significantly lower reaction rate. However, it was clear that after 30 hours the reaction rate was decreasing. It may be due to the inhibition of enzyme activity by excess methanol or glycerol which was a byproduct of reaction. The optimum reaction duration was selected as 30 hours.

3.3. Effect of pH

The manufacturers of ROL suggested a pH just above 7 would be ideal for the enzyme activity. So to know about the effect of pH on reaction experiments were conducted with pH varying between 6 and 8. Sodium dihydrogen phosphate (NaH$_2$PO$_4$ - Molecular weight – 138 g) and disodium hydrogen phosphate (Na$_2$HPO$_4$ - Molecular weight – 142 g) were the buffer salts used for preparing buffer solution. After preparing 0.1M solution of each salt they were mixed according to the buffer chart to obtain required pH.

Figure 2 shows the effect of pH on percentage of biodiesel conversion. Fixing of pH adversely affected the reaction. When pH was fixed at 8 conversion was very low. The natural trend of the reaction was to decrease the pH of the mixture, ie pH of the reaction mixture was shifted to acidic region. When pH was fixed at 8 this natural shift did not occur which led to lower reaction rate.

<table>
<thead>
<tr>
<th>pH</th>
<th>NaH$_2$PO$_4$(ml)</th>
<th>Na$_2$HPO$_4$(ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>88</td>
<td>12</td>
</tr>
<tr>
<td>7</td>
<td>42.3</td>
<td>57.7</td>
</tr>
<tr>
<td>8</td>
<td>6.8</td>
<td>93.2</td>
</tr>
</tbody>
</table>

Fig. 2. Variation of percentage of conversion against pH of reaction mixture
3.4. Effect of acyl acceptors

Most commonly used acyl acceptors for biodiesel production are short chain alcohols like methanol and ethanol as the commercial availability of these alcohols are more. But alcohol present in the reaction mixture will inhibit the activity of enzyme which will reduce the extent of reaction. Acetates are one of the good substitutes for short chain alcohols as they do not have inhibitory action on enzymes [17]. Acyl acceptors used in the present experiments were ethyl acetate and methanol. Among them ethyl acetate gave better results for biodiesel production from rubber seed oil even at higher molar ratios, which indicates that ethyl acetate does not have any inhibition action on ROL. Figure 3 shows effect of acyl acceptors on reaction. Molar ratio for both methanol and ethyl acetate was 1:4. After 24 hours ethyl acetate gave 27.9% of conversion where methanol gave only 22.05% of conversion. To avoid inhibition by methanol 3 step addition was followed. Still the result showed that stepwise methanol addition was not sufficient to eliminate enzyme activity inhibition.

![Fig. 3. Percentage of conversion vs acyl acceptors](image)

3.5. Effect of molar ratio

Another important variable in transesterification is molar ratio. For the conversion of one mole of triglyceride to ester three moles of alcohol is needed. Since molar ratio 1:3 is just sufficient for theoretical reaction, most of the researchers suggested a higher molar ratio value for maximum biodiesel conversion [1]. Molar ratio of samples were

![Fig. 4. Effect of molar ratio on percentage of conversion (a) methanol (b) ethyl acetate](image)
varied as 1:4, 1:7, 1:9, 1:11 for ethyl acetate and 1:4, 1:5 for methanol. The variation in biodiesel conversion percentage is plotted in Fig. 4. With increase in molar ratio of ethyl acetate from 1:4 to 1:11 the percentage of conversion was increased from 29.8 to 33.3. This shows that ethyl acetate does not have inhibition effect on ROL activity. In case of methanol when molar ratio was increased, a reduction in conversion efficiency was observed which shows the inhibition of enzyme reactivity by methanol.

4. Conclusion

Biodiesel production from high FFA rubber seed oil using enzyme as catalyst was investigated in this study. The lipase Rhizopus Oryzae was the considered potential lipase for biodiesel production. The reaction was mainly influenced by catalyst concentration, time duration, acyl acceptor used and molar ratio.

With increase in catalyst concentration it is observed that conversion percentage increases, but considering the high cost of enzyme increase in concentration above 10(w/w) % of oil is not advisable as it will seriously affect the production cost. After 30 hours it was noticed that the reaction rate was decreased irrespective of the enzyme quantity.

Even though pH was considered as a dependent variable for biodiesel conversion in the study, it did not show any positive result in reaction. It was observed that pH of the reaction mixture becomes slightly acidic as the reaction progresses. When the pH of the mixture was fixed at 8 (slightly basic) the conversion was poor. So it was concluded that fixing of pH using buffer decelerates the reaction.

In the production of biodiesel enzyme activity was seriously influenced by traditional acyl acceptor methanol. When the molar ratio was increased from 1:4 to 1: 5 a reduction in conversion was observed which showed the inhibitory action of methanol on activity of enzyme. However ethyl acetate an alternate acyl acceptor showed no negative effect on activity of enzyme. Even at higher molar ratios (1:11) percentage of conversion was good enough for reaction which confirms that ethyl acetate did not inhibit the activity of ROL. The additional attractive thing is that use of ethyl acetate in transesterification gives triacetylgllycerol as byproduct which has worthier than glycerol which is the byproduct of methanolysis. When compared with previous works using Candida Antartica Lipase B [7] and steapsin [13] the conversion percentage is very low. Therefore ROL cannot be considered as an advisable lipase for transesterification of high FFA contained rubber seed oil.

References