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A comparative study between chemical and enzymatic transesterification of high free fatty acid contained rubber seed oil for biodiesel production

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Abstract: The choice of a paramount method for biodiesel production has significance as the demand of alternative fuels like biodiesel is growing rapidly. In the present study, experimental results from chemical-catalysed as well as enzyme-catalysed methods were compared using common influencing parameters such as oil/alcohol molar ratio, catalyst concentration and reaction duration. Requirement of certain solvents to enhance the reaction rate was explained in the enzyme-catalysed transesterification reaction. Biodiesel conversion of more than 90% was attained for chemical-catalysed transesterification, whereas the conversion rate was 85% for enzyme-catalysed method. This gives the indication of further refinement in the enzyme-catalysed transesterification process. The influencing parameters and absolute results of the analysis give the impression of superiority of enzymatic transesterification method for biodiesel production from high free fatty acid-contained rubber seed oil.

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1. Introduction

Production of biodiesel to replace petroleum-refined diesel for the purpose of reduced pollution and sustainability is not a novel technique. The most established method for biodiesel production is chemically catalysed transesterification. But the cost of production and complexity in the processes lead to the need of alternate methods for biodiesel production. Enzyme-catalysed transesterification is a relatively new method for biodiesel production. In this method, enzymes which have the capability to catalyse transesterification of oils/fats (lipids) commonly known as lipases are used.

Chemical transesterification depends highly on the purity of the reactant oil especially free fatty acid (FFA) content. Canakci & Van Gerpen, 2001 reported from their experiments that in most of the cases, transesterification reaction stops at an acid value above 2 mgKOH/g while reacting on high FFA feed stocks. An acid esterification pre-treatment is mandatory for high FFA-contained vegetable oils. If the FFA content is too high, one-step acid esterification with sulphuric acid (H₂SO₄) may not be sufficient which may lead to two- or three-stage pre-treatment process. The pre-treated oil will then undergo alkaline transesterification for biodiesel production.

Enzymes are biological catalysts which accelerate the rate of a chemical reaction without undergoing a permanent change in the structure. Lipase is an enzyme that catalyses the breakdown or hydrolysis of fats (lipids) (Svendsen, 2000). Lipases fall in a subclass of esterases. Lipases (triacylglycerol acyhydrolases, EC 3.1.1.3) constitute various families of enzymes which are produced by animals, plants and micro-organisms. Since their bulk production is easier, commercialisation of microbial lipases and their involvement in enzymatic biodiesel production are more common than animal and plant ones. The microbes that have been suggested for biodiesel production include Aspergillus niger, Candida cylindracea, Candida rugosa, Candida antarctica, Chromobacterium viscosum, Pseudomonas cepacia, Pseudomonas fluorescens, Rhizopus oryzae. (Gog, Roman, Toşa, Paizs, & Irimie, 2012). Among these, C. antarctica displayed highest activity in methanolysis and ethanolysis reactions. C. antarctica in solvent-free environment resulted in above 90% conversion in majority of literature (Gog et al., 2012; Ho, Ngoc, Hyun, Mi, & Koo, 2007; Luque & Cervero, 2014; Modi, Reddy, Rao, & Prasad, 2007; Tupu, Jae, Marquis, Adesina, & Rogers, 2013).

Since lipases are insensitive to FFA, the oil does not require a pre-treatment and both the esterification and transesterification take place simultaneously to produce biodiesel directly. In addition to this, moisture content in the raw oil does not degrade the reaction, in fact will enhance the reactivity of the enzyme. However, the extended period of reaction and cost of enzymes restrict the wide acceptance of enzymatic transesterification for biodiesel production. Lipase immobilisation technology provides a number of important benefits including: (a) enzyme reuse, (b) easiness in separation of product from enzyme and (c) decrease in the inhibition rate. The process parameters are optimised in enzymatic transesterification process to make it a viable method for biodiesel production.

2. Materials and equipment

Crude rubber seed oil (RSO) was purchased from Pavalm & Co, Virudhunagar, Tamil Nadu, where the oil was extracted by expelling cured (de-shelled and dried) rubber seeds. Analytical grade methanol was used for transesterification. The bio-catalyst (enzyme) used was C. antarctica lipase B (Fermase CALB 10,000). Twenty-five grams of the lipase was supplied by Fermenta Biotech Ltd. Thane free of cost. PLU activity of the enzyme was specified as NLT (not less than) 10,000 U/g dry (One PLU enzyme unit corresponds to synthesis of 1 μmol of propyllaurate per minute per gram of immobilised enzyme at 60°C from lauric acid and 1-propanol). The enzyme was immobilised on polyacrylate beads and appeared as white- to creamish-white-coloured granular form.
Toluene, Isopropyl alcohol, n-Hexane, t-Butanol, Chloroform (all analytical grade) and deionised water were used as reagents for FFA estimation of oil and purification of biodiesel. A magnetic stirrer with hot plate was used as the mixing and heating equipment for chemical transesterification. The temperature was measured continuously with mercury column thermometer during the process.

In enzymatic transesterification, the reaction mixture was shaken in an incubated shaker in order to keep the stability of enzyme in the immobilisation medium. The incubator temperature was maintained as constant at 37°C throughout the experiments. The fatty acid compositions of RSO as well as the final biodiesel production yield were analysed by GC–MS technique in Care Keralam Ltd., Koratty, Kerala. The molecular formula of RSO was arrived as $C_{18}H_{32}O_{32}$ and molecular weight as 278. The properties of the oil are shown in Table 1.

The other equipment and instruments used include Centrifuge, Micro-Weighing balance, Micro pipette, Brookfield viscometer, Bomb calorimeter, Pensky Martin apparatus and Thermometers.

### 3. Chemical transesterification

In transesterification, reaction oil and an alcohol react together in which the glycerol backbone from the triglyceride molecule will be separated. The separated fatty acids will undergo reaction with alcohol to become a mixture of fatty acid alkyl esters which is termed as biodiesel. This reaction requires an elevated temperature of about 300°C with increased pressure (to avoid boiling of alcohol). Chemical catalysts will reduce the reaction temperature to a range of 50–60°C at normal atmospheric pressure condition. The commonly used chemical catalysts are potassium hydroxide (KOH) and sodium hydroxide (NaOH).

![Chemical transesterification diagram]

#### 3.1. Influence of free fatty acids

FFAs are the chain-structured molecules which are separated from the glycerol backbone and usually have even number of carbon atoms. These FFAs do not take part in transesterification reaction and will make intermediate compound such as soap which has the capability to inhibit the biodiesel production reaction. The saponification characteristics of raw oil make it impractical to use in

| Property                        | Value  |
|---------------------------------|--------|
| Fatty acid composition (%)      |        |
| (1) Palmitic acid $C_{16:0}$    | 10.2   |
| (2) Stearic acid $C_{18:0}$     | 8.7    |
| (3) Oleic acid $C_{18:1}$       | 24.6   |
| (4) Linoleic acid $C_{18:2}$    | 39.6   |
| (5) Linolenic acid $C_{18:3}$   | 16.3   |
| Specific gravity                | 0.91   |
| Viscosity (mm²/s) at 30°C       | 59.7   |
| Flash point (°C)                | 210    |
| Acid value (mgKOH/g)            | 82     |
biodiesel production. The researchers through their vast experience claim that FFA content less than 2% is indeed to go for the transesterification reaction with alkali catalysts (Bharathiraja et al., 2014; Ramadhas, Jayaraj, & Muraleedharan, 2005). Therefore, a pre-treatment process (acid esterification) should be done on the raw oil before proceeding to alkali transesterification for biodiesel production.

3.2. Acid esterification
Standard AOCS titration method (Lubrizol, 2013) was adopted to determine acid value of the oil (percentage of FFA is half the acid value). In acid esterification, the FFA will react to alcohol (methanol) in the presence of sulphuric acid (H₂SO₄) so that it will be converted into alkyl ester and water.

\[
\text{FFA} + \text{Methanol} \xrightarrow{\text{Acid catalyst}} \text{Methyl ester} + \text{Water}
\]

FFA content of RSO was very high (42%) so that one-stage esterification was insufficient to reduce it to the desired level. Canakci and Van Gerpen (2001) reported from their experiments that in most of the cases, acid-catalysed reaction stops at an acid value above 2 mgKOH/g while reacting on high FFA feed stocks. Two-step acid esterification technique was used in the present study to reduce the acid value to the desirable range.

Different sets of experiments were conducted with varying oil to alcohol molar ratio and acid catalyst concentration. In each run, 200 ml of RSO was filled in the flask of 500-ml capacity and pre-heated to 50°C. Pre-determined amount of methanol was then added to the oil and stirring was started. After a few minutes, acid catalyst was added to the mixture and the stirring was continued. In the first step, reaction duration was 1 h, keeping the temperature at 45 ± 5°C throughout and the acid catalyst concentration at 1% (v/v). After the reaction, the mixture was kept in a separating funnel for 24 h. Water and methanol mixture along with sulphuric acid content moved to top. There was a layer of residue settled at the bottom containing colouring pigments, gum forming agents, wax, etc.

The second stage of acid esterification was carried out with different molar ratios keeping other parameters same as in the first stage. Three-stage reactions were also carried out with lower methanol ratio, and it was observed that the increase in methanol ratio caused a significant reduction in acid value. The residue and methanol water mixture were separated after the reaction. Five sets of selected experimental results are presented in Table 1. Use of an overall molar ratio of 80% v/v methanol and 2% v/v concentration of H₂SO₄ in two stages is found to give optimum results. The result obtained from pre-treatment process shows relatively less oil–methanol ratio in comparison with the work done by Satyanarayana and Muraleedharan (2010). Experimental trials were also carried out with varying acid catalyst concentration with optimum alcohol to oil molar ratio.

3.3. Effect of acid catalyst concentration
Ahmad, Yusup, Bokhari, and Kamil (2014) conducted experiments with 10 wt% H₂SO₄ in which 45% FFA-contained RSO was reduced to 0.82% in single-step acid treatment. Experiments were conducted with varying acid catalyst amount from 1% to 10% with favourable methanol ratio in the first-stage reaction. Even if the FFA content reduced continuously with increase in H₂SO₄, it did not reach to the desired value (2%) even at 10% v/v (refer Figure 1). Saponification occurred up to a FFA content of 3% on water washing. Therefore, multistage acid esterification was suggested as preferred. The catalyst concentration of 1% w/w was recommended for the first-stage reaction after considering the molar ratio and number of reaction stages (see Table 2).

3.4. Effect of molar ratio
The amount of alcohol used in both acid esterification and alkaline transesterification plays a significant role in reaction rate and overall production cost of biodiesel. Experiments were carried out with different methanol to oil molar ratios of 0.20, 0.40, 0.50, 0.60 and 0.65. A significant reduction in acid value was observed by increasing the percentage of oil–alcohol ratio up to 0.50. The trend of FFA
Figure 1. FFA variation with acid catalyst concentration at 50% v/v methanol in oil (after first-step pre-treatment).

Figure 2. FFA reduction with methanol ratio with catalyst 1% w/w (after first-step pre-treatment).

Table 2. Absolute values of FFA content in the oil (temperature 45 ± 5°C, 1-h reaction, 1% v/v H₂SO₄ in each step)

| Set No. | Reaction stages | Molar ratio (% v/v) | Titration end point value (ml) | FFA (%) |
|---------|-----------------|---------------------|--------------------------------|---------|
| 1       | 1st stage       | 20                  | 29                             | 16.29   |
|         | 2nd stage       | 20                  | 15                             | 8.41    |
|         | 3rd stage       | 20                  | 5                              | 2.8     |
| 2       | 1st stage       | 40                  | 20                             | 11.22   |
|         | 2nd stage       | 30                  | 9                              | 5.04    |
|         | 3rd stage       | 20                  | 3                              | 1.68    |
| 3       | 1st stage       | 50                  | 14                             | 7.85    |
|         | 2nd stage       | 30                  | 3.6                            | 2.02    |
| 4       | 1st stage       | 60                  | 16                             | 8.97    |
|         | 2nd stage       | 20                  | 4                              | 2.44    |
| 5       | 1st stage       | 65                  | 15                             | 8.41    |
|         | 2nd stage       | 20                  | 4.5                            | 2.52    |
reduction with molar ratio in first stage of pre-treatment is shown in Figure 2. It was noted that after the limit of 0.50, use of excess alcohol did not give any significant reduction of FFA content.

The second step of acid esterification was done for further reduction of FFA in the oil. Catalyst concentration was kept as 1% v/v for each set of second-step esterification. The molar ratios of each step with resultant FFA content are listed in Table 2.

4. Alkaline transesterification
Alkaline transesterification was carried out after the completion of two-stage pre-treatment process. The base catalyst used was KOH (85% assay) and molar ratio of oil to methanol was kept as 1:9 in each experiment. Properly mixed solution of KOH and methanol of required quantity was added to 150 ml of treated and preheated oil (50°C) and was stirred well. The temperature was maintained at 55 ± 3°C throughout the experiment for 40-min reaction time. The previous studies and literatures showed that there was no significant increase in biodiesel yield with increase in reaction time above 40 min (Satyanarayana & Muraleedharan, 2010). After the completion of the reaction, products were allowed to settle for 24 h. Glycerine was removed when it was settled at the bottom. The top layer (biodiesel) was purified by washing 3–5 times with demineralised water at room temperature.

4.1. Effect of base catalyst
Base catalyst concentration was varied from 0.5 wt% to 2 wt% and the result revealed that maximum yield of 90% was obtained with a base catalyst concentration of 1.5 wt% (refer Figure 3). The conversion efficiency did not improve after the limit of 1.5 wt% KOH addition. Emulsion was observed with lower catalyst concentrations (0.5–1 wt%) due to insufficient catalyst amount to completely neutralise the entire FFA resulting in saponification reaction. The soap content in the biodiesel made emulsion during water washing.

An excellent medium to break the emulsion was sodium chloride (NaCl) solution. NaCl solution of 50% by volume was added to the emulsion and was heated to 60°C and stirred. It has been observed that the biodiesel separated from the emulsion solution is coming out at the top. The entire chemical transesterification process including the two-step acid treatment and alkaline transesterification is summarised as a flow diagram shown in Figure 4.
5. Enzymatic transesterification

The use of enzymes as catalysts for transesterification of high FFA-contained oils is a promising technique as the enzymes are insensitive to FFA. Pre-treatment process was eliminated in this method. Different sets of experiments were carried out to study the yield of biodiesel with different molar ratio, enzyme concentration, type of solvent and duration of reaction. RSO of 10 ml was used in each experimental run. Reactions were carried out in a conical flask of 100-ml working volume. The enzyme was weighed according to the wt% of oil in each set and added to the RSO. Methanol was measured by micropipette and added to the oil stepwise (three steps) at equal time interval. The reaction mixture with methanol, oil and enzyme was shaken in an incubated shaker at 170–200 rpm keeping the temperature at 37°C. After 24 h, reaction enzyme was separated out from the reaction mixture by filtration. In order to reduce time for experimental process, the mixture of biodiesel and glycerol produced after the reaction was centrifuged at 5,000 rpm for 15 min. Glycerol was settled at the bottom of the centrifuge tube and was removed. The separated mixture was heated above 100°C to remove unreacted methanol and water droplets. The enzyme separated was washed with distilled water and t-butanol, dried and stored under the required temperature (below 8°C) for further use.

5.1. Effect of methanol ratio

Influence of methanol/oil molar ratio was measured by varying it between 2 and 6. Even though the theoretical molar ratio required is 3, most of the researchers suggested a higher molar ratio in order to avoid the insufficiency of alcohol for the reaction (Gog et al., 2012; Yan et al., 2014). Sufficient alcohol may not be available for the reaction due to the traces of water and other liquid impurities in the oil which will be dissolved in it (Ramadhas et al., 2005). For each set of experiments, the enzyme concentration was maintained as 10% w/w of oil and no solvent was added to enhance the reaction rate. The trend of molar ratio against biodiesel conversion efficiency is plotted in Figure 5. Even if the conversion efficiency of molar ratio 6 was slightly higher than that of molar ratio 4, upon optimising considerations, molar ratio 4 was taken as recommended value.

5.2. Effect of enzyme concentration

The variation of product yield and kinematic viscosity was analysed with different enzyme concentrations. Enzyme weights between 5 and 12 were used in different sets by keeping the methanol/oil molar ratio at optimum value (molar ratio 4). According to Sanchez and Vasudevan (2006) and Chattopadhyay, Karemore, Das, Deysarkar, and Sen (2011), increase in reaction rate is directly proportional to enzyme concentration. In the present experiments, the biodiesel conversion ability was continuously increased but reduced beyond 10 wt%. The resulted viscosity value of the products is plotted in Figure 6. The excess solid enzyme available in the reactive mixture had the ability to limit
glycerol separation due to the non-interaction between methanol and oil. Similar result was reported by Shah and Gupta (2007) such that the maximum conversion was obtained at 10% w/w of oil.

5.3. Effect of solvent addition

Lower chain alcohols have the tendency to inhibit the reactivity of the lipases. The addition of solvents to the reaction mixture will enhance the enzyme activity. The solvents prevent the inhibition of enzyme activity by dissolving the alcohol and give the flexibility to the enzyme. In addition to this, glycerol produced during the early stages of transesterification has adverse effect on biodiesel production by accumulating on the immobilisation support (Moreno-Pirajan & Giraldo, 2011). Water and t-butanol were the commonly used solvents for hydrolysis reaction of oils and fats (Luque, Cervero, & Alvarez, 2014). According to Sun, Yu, Curran, and Liu (2012), lipases act efficiently at oil–water interface. Also t-butanol has greater ability to dissolve glycerol. Addition of small amount of water (not more than 5% w/w of oil) is preferable in lipase-catalysed transesterification (Gog et al., 2012; Yan et al., 2014). Different sets of experiments were done with and without solvent additions keeping other parameters optimum. The biodiesel conversion rate and kinematic viscosity of the products of
reaction are expressed in the following graph (Figure 7). The absolute values of conversion percentage were mentioned in the line diagram. The downward-directed bar diagrams represent the reduction in viscosity value from the viscosity of raw oil and the absolute kinematic viscosity values were represented in cSt.

A maximum conversion percentage of 79.45 resulted in the experiments with no solvent addition. The addition of 7.5% w/w t-butanol has a reduced conversion rate, whereas the addition of 5% w/w of water significantly increased the conversion rate and reached to a maximum of 84.69%. Both the solvents were added in three steps at the time of methanol addition. A separate experiment was conducted by the addition of both the solvents each with 5% w/w of oil. The second solvent (t-butanol) was added after a time gap of 8 h from the beginning of reaction. The results showed that there was no improvement in conversion efficiency with addition of t-butanol. It can be inferred that the effect of enzyme activity inhibition by the presence of glycerol was insignificant in low-quantity enzymatic RSO biodiesel production. Therefore, the best solvent medium for enzyme transesterification for RSO was identified as water with methanol.

5.4. Effect of reaction duration
Since the production duration of biodiesel in enzyme-catalysed method is longer than chemical method, determination of optimum reaction duration is highly important in enzymatic transesterification. Experiments were performed with different durations of reaction. Reaction time was extended up to 43 h keeping other parameters optimum. It has been recorded that the maximum conversion is obtained for a duration of 30 h (Figure 8) after which the overall conversion percentage was less. This may be due to the reverse reaction in the transesterification process.

6. Statistical analysis of influencing factors for enzymatic transesterification
Since parameter optimisation was done based on literature reviews, statistical testing was avoided in chemical transesterification reaction method. Being a novel method of biodiesel production, one statistical model was formulated to study the influence of parameters on biodiesel production yield in enzymatic transesterification. The $2^3$ full factorial model was adopted for the study. Only the concentration parameters were considered for analysis. The factors taken for analysis were enzyme concentration (E), molar ratio (M) and solvent addition (S). The software tool used was Design-Expert® Version 10. Both enzyme concentration and molar ratio were considered as numerical variables having limits 5, 10 wt% and 4, 5 v/v%, respectively. Solvent addition term was entered as categorical variable with “Yes” (5 wt%) and “No” (0 wt%). Table 3 shows the details of ANOVA.
From the table, \( p \)-value is less than 0.0500 which indicates that model terms are significant. In this case, \( E \) and \( S \) are significant model terms and \( M \) is insignificant. The table also infers that interactive effect of variables \( E-M \) and \( E-S \) is insignificant and \( E-M-S \) is too small enough to remove from normal plot. There is only 0.50% chance that an \( F \)-value could occur due to noise. The regression equation in terms of actual values at solvent added condition (\( S = \text{Yes} \)):

\[
(Y)^{3,32} = -8679.24 + 4805.13 \times E + 3528.34 \times M - 566.8 \times E \times M
\]

### 7. Comparison between chemical transesterification and enzymatic transesterification

Based on the experimental results, the important parameters on biodiesel production process through transesterification can be summarised (refer Table 2). It is important to recommend the suitable method for the biodiesel production from high FFA-contained RSO. Table 4 gives a comparison between chemical and enzymatic transesterification processes with respect to important aspects.

Other than reaction duration and catalyst concentration, all other parameters give superiority to enzymatic reaction. Use of immobilised enzyme catalyst can compensate higher catalyst consumption problem since the enzyme can be reused for several batch operations. Even though the chemical reaction duration is quite less in comparison with enzymatic reaction, the overall biodiesel...
production period was comparable. This is due to the repeated water washing and separation steps needed due to high FFA content of oil in chemical transesterification method.

8. Conclusion
Enzymatic transesterification being the newly identified technique for biodiesel production needs further refinements to meet the replacement of the established method of chemical transesterification. On comparison of the two methods, it can be concluded that enzyme-catalysed reaction is superior to chemical method. Enzyme-catalysed transesterification has given more pure biodiesel as well as glycerol than that from chemical method. Still, the conversion percentage of enzyme-catalysed transesterification is less. The increased reaction duration of enzymatic transesterification restricts the production process to batch production method. High-level mixing methods such as ultrasonic vibration method can be adopted to increase the rate of reaction in enzymatic transesterification. However, upon absolute measurement of total process duration including pre-treatment stages and gravity separation, chemical transesterification production of high FFA-contained RSO is lengthier. The traces of highly reactive acid and base contents in the reaction products need several levels of water washing and neutralisation in chemical method, thereby increasing the number of downstream processes. The present experiments also throw light on identification of the potential of underutilised RSO feedstock for biodiesel production.

| Sl. No. | Property | Chemical transesterification | Enzymatic transesterification |
|---------|----------|-----------------------------|-------------------------------|
| 1       | Catalyst | KOH                         | Candida antarctica lipase B   |
| 2       | Oil/methanol molar ratio | 1:9 | 1:4 |
| 3       | Catalyst concentration | 1% w/w | 10% w/w |
| 4       | Sensitivity to FFA | Highly sensitive, direct reaction leads to saponification | Insensitive to FFA content in the oil |
| 5       | Necessity of pre-treatment | Two-stage acid esterification (pre-treatment) is needed to meet the required oil quality | No need of pre-treatment |
| 6       | Reaction period | 2–3 h (including pre-treatment) | Minimum 24 h |
| 7       | Sensitivity to water content in oil | Highly sensitive, need to remove entire water particles before the start of reaction | Presence of water (up to 5% w/w) can improve the rate of reaction |
| 8       | Solvent addition | No solvent needed | Addition of solvents improves the reaction rate significantly |
| 9       | Biodiesel production yield | More than 90% | About 85% |
| 10      | Purity of by-product glycerol | Impure and needs further purification steps | Glycerol separated is pure |

**Table 4. Comparison of important parameters in the transesterification reactions**

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