

# Production of Biodiesel from High Acid Value Waste Cooking Oil Using an Optimized Lipase Enzyme/Acid-Catalyzed Hybrid Process

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**Abstract:** The present study is aimed at developing an enzymatic/acid-catalyzed hybrid process for biodiesel production using waste cooking oil with high acid value (poor quality) as feedstock. Tuned enzyme was prepared using a rapid drying technique of microwave dehydration (time required around 15 minutes). Further enhancement was achieved by three phase partitioning (TPP) method. The results on the lipase enzyme which was subjected to pH tuning and TPP, indicated remarkable increase in the initial rate of transesterification by 3.8 times. Microwave irradiation was found to increase the initial reaction rates by further 1.6 times, hence giving a combined increase in activity of about 5.4 times. The optimized enzyme was used for hydrolysis and 88% of the oil taken initially was hydrolyzed by the lipase. The hydrolysate was further used in acid-catalyzed esterification for biodiesel production. By using a feedstock to methanol molar ratio of 1:15 and a sulphuric acid concentration of 2.5%, a biodiesel conversion of 88% was obtained at 50 °C for an hour reaction time. This hybrid process may open a way for biodiesel production using unrefined and used oil with high acid value as feedstock.

**Keywords:** Acid catalyst, pH tuned enzyme, Lipase-*Candida rugosa*, Three phase partitioning, Microwave drying and Hybrid process.

## Introduction

Exploring new renewable resources for energy needs has been gaining great importance in recent years due to mounting crude oil prices, an irrevocable decrease in oil reserves, and increasing environmental degradation. Biodiesel, which is a product of the transesterification of fats and oils by lower alcohols like methanol, is becoming increasingly

popular as a straight motor fuel or as biodiesel blends through mixing with various levels of fossil diesel, in developing as well as developed countries<sup>1,2</sup>. The ability to use biodiesel directly in diesel engines with little or no modification, with similar fuel economy, and with reduced greenhouse gas emission are the primary advantages of biodiesel, in addition to its qualities of being non-toxic and biodegradable. Since much of the carbon found in the oils and fats used to create the fuel originated from CO<sub>2</sub> in the atmosphere, biodiesel is considered to contribute much less to global warming than conventional diesel. Vegetable oils (soybean oil in the US, rapeseed oil in Europe, and palm oil in tropical countries), off-quality oils, used cooking oils and animal fats have been used as raw materials for biodiesel production using transesterification<sup>3</sup>. Transesterification of these raw materials can be carried out in the presence of a catalyst, such as alkali<sup>4</sup>, acid<sup>5</sup>, or an enzyme<sup>6,7</sup>. In alkali-catalyzed transesterification, sodium hydroxide and potassium hydroxide are commonly employed as catalysts. This method is the most popular process for biodiesel production, but it requires strict feedstock specification. Only highly refined vegetable oils should be used as feedstock to produce biodiesel by this method. Furthermore, the water and free fatty acid (FFA) contents in the reaction system should be less than 0.1 and 0.5%, respectively<sup>8</sup>. At high water contents, hydrolysis will become the dominant reaction and leads to a decline in biodiesel yields. In addition, the use of oils with higher FFA contents in alkali-catalyzed transesterification results in the formation of soaps and causes difficulties in the purification of biodiesel, thereby increasing the overall production cost. Alternatively, the transesterification of oils to biodiesel has been carried out in the presence of acid catalysts, such as sulphuric acid, hydrochloric acid, and organic sulphonic acid. The acid-catalyzed transesterification can tolerate high FFA and water contents, and due to this fact, unrefined oils can be used as feedstock for biodiesel production via acid catalysis. However, higher reaction temperature (100 °C) and longer reaction time (48 h) are required for achieving higher biodiesel conversions<sup>8</sup>. Besides, both alkali and acid catalyzed transesterification processes produce large amounts of wastewater, which is of environmental concern. The use of enzymes for the transesterification of oils eliminates the inherent problems associated with the use of chemical catalysts. However, enzymes are easily deactivated in the presence of polar compounds like water, methanol, and glycerol due to the low solubility of the polar compounds in the oil phase<sup>9</sup>. The key step in enzymatic processes lies in successful improvement of the efficiency of the enzymatic process and the stability of immobilized enzymes. Additionally, the high cost of immobilized enzymes makes this biodiesel more expensive than that produced by chemical catalysis. The repeated use of the immobilized enzyme and the high market price of pure glycerol produced during the enzyme-mediated transesterification, in turn, can result in a considerable reduction in the cost of production of biodiesel<sup>10</sup>.

Recently, it was observed that three phase partitioning (TPP) of Proteinase K resulted in enhancement of its catalytic power in aqueous buffers<sup>11</sup>. The process, originally described as a bio-separation strategy, consists of adding less than 'salting out' amount of ammonium sulphate and *t*-butanol to the aqueous solution of the enzyme. The X-ray diffraction pattern of Proteinase K, after being subjected to TPP, showed unusually high 'B-factor', signifying overall increase in the flexibility of the protein molecule<sup>11</sup>. Three phase partitioning (TPP) shows sufficient promise for obtaining enzymes with better catalytic power in both aqueous and non-aqueous media and appears to be a useful method to improve the purity and efficiency of enzymes. The enzyme, after being subjected to TPP, showed greater activity in organic solvents. It was hoped that such a TPP-treated enzyme with a more flexible structure would have higher catalytic efficiency<sup>12</sup>. The use of microwaves irradiation has become a fairly routine technique to enhance reaction rates of chemical reactions. One does not have to worry about thermal inactivation while using microwaves if the medium is nearly anhydrous.

Enhancements of reaction rates in the microwave-assisted esterification were observed with un-tuned, pH-tuned and salt-activated enzymes<sup>13</sup>. In this study, we will employ microwave irradiation as a dehydration method (instead of lyophilisation) for pH tuning. The tuned enzyme will be further subjected to three phase partitioning (TPP) and microwave irradiation to further enhance its catalytic activity.

Contrary to the single-step biodiesel production process using transesterification of oils, the use of an enzymatic/acid-catalyzed hybrid process is proposed for the production of biodiesel from waste cooking oil in our study. In the first step, low value waste cooking oil with high free fatty acid value (more than 5%) was hydrolyzed in the presence of lipase which had been immobilised to chitosan beads using a binary method<sup>14</sup>. In the second step, the feedstock consisting of FFA, mono- di- and tri-glycerides obtained from the hydrolysis was esterified with methanol in the presence of an acid catalyst to produce biodiesel. This hybrid process was chosen for our study due to several factors. First, enzymatic hydrolysis can tolerate oils with high FFA contents; therefore, unrefined oils with high FFA contents can be used as feedstock for biodiesel production. Also, when compared with acid catalyzed transesterification, the enzymatic/acid-catalyzed hybrid process uses milder reaction conditions. Finally, as opposed to enzymatic transesterification, the enzymatic/acid catalyzed hybrid process can avoid inactivation of the immobilized enzyme by polar compounds and increase biodiesel yields.

## Experimental

Lipase (*Candida rugosa*) was purchased from HmbG Chemicals. tris-HCl (was a product of CalBiochem. Ammonium sulphate and *t*-butanol were purchased from Merck. *p*-Nitrophenyl palmitate (4-nitrophenyl palmitate) and *p*-nitrophenol (4-nitrophenol) were purchased from Sigma-Aldrich Corporation. The solvents were purchased from Aldrich in the anhydrous form (Sure/Seal bottles, water content below 0.005%). All other chemicals were of analytical grade. Microwave irradiation were carried out in a domestic microwave oven (National Model NN-GD 576 M). Aqua Lab model series 3, water activity meter was used to determine the water activity.

## Procedures

### *pH- tuned enzyme*

The native, lipase enzyme (200 mg) was dissolved in 10 mL of 0.02 M tris-HCl, pH 7.8 and then subjected to several short bursts of microwave irradiation at frequency of 2.45 GHz, at power output of about 100 W for 10 seconds. The sample was then cooled. This ramp/cool cycle was repeated 12-15 times until the sample was dry. This was referred to as pH-tuned enzyme.

### *Three phase partitioning (TPP) and pH tuning of enzyme*

Lipase solution (9 mL, 3 mg solid native enzyme/mL of 0.02M tris-HCl, pH 7.8), was saturated with 50% (w/v) solution of ammonium sulphate. This was followed by addition of 18 mL of *t*-butanol (1:2, v/v, ratio of the enzyme solution to *t*-butanol). The solution was vortexed and allowed to stand at 25 °C for 1 h. The solution was then centrifuged (2000 x *g*, 10 min). The lower aqueous and upper organic layers were separated using a pasteur pipette. The interfacial precipitate was dissolved in 5 mL of 0.02 M tris- HCl, pH 7.8 and dialyzed against the same buffer for 16 h at 4 °C<sup>12</sup>. After being dialyzed, the enzyme solution was subjected to microwave irradiation for 10 second at frequency of 2.45 GHz, a power output of about 100 W. The sample was then cooled. As mentioned previously, this ramp/cool cycle was repeated 12-15 times until the sample was dry. This was referred to as TPP-pH tuned lipase.

*Assay for lipase activity using p-nitrophenylpalmitate (pNPP) as substrate*

The activity of lipase in low water organic media was monitored by following the hydrolysis of *p*-nitrophenyl palmitate in water-saturated *n*-heptane (using varying water saturation from 0 to 1.0% water activity,  $a_w$ )<sup>15</sup>. The release of *p*-nitrophenol yields a yellow colour which was measured spectrophotometrically at 410 nm (25 °C).

*Lipase assay reagent*

Reagent A consisted of 0.0667 g of Gum Arabic (Acacia tree) dissolved in tris-HCl buffer (12 mL, 250 mM, pH 7.8) containing 48 mL of distilled water. Thereafter 0.267 g of sodium deoxycholate was added and dissolved in the solution. Reagent B consisted of either 24 mg of *p*-nitrophenyl palmitate dissolved in 8 mL propan-2-ol at 37 °C. Reagent B (1 mL) was added to 9 mL of reagent A with rapid stirring for approximately 20 seconds<sup>16</sup>.

Enzyme (10 mg) was added to 900  $\mu$ L of the lipase assay reagent and the reaction mixture was incubated at 40 °C for 10 min. The reaction was terminated by adding 2  $\mu$ L of 0.5 M EDTA. The activity was assayed by detecting the product, *p*-nitrophenol, spectrophotometrically at 410 nm with a GENESYS 10 UV-Vis spectrophotometer (Thermo-Fisher) at 25 °C against an enzyme-free blank. The molar extinction coefficient of *p*-nitrophenol at pH 7.8 was calculated as 15.1 M<sup>-1</sup>cm<sup>-1</sup>. One unit of enzyme activity was defined as the quantity of enzyme required to liberate one  $\mu$ mole of *p*-nitrophenol per minute under the above conditions.

*Acid number determination*

High fuel acidity is associated with corrosion and engine deposits, particularly in the fuel injectors. The acid number or acid value of edible oils or their corresponding esters indicates the quantity of free fatty acids (FFA) and mineral acids (negligible) present in the sample. According to ASTM D 664 and EN 14104, the acid number is expressed in milligrams (mg) potassium hydroxide (KOH) required to neutralize 1 g of sample. In order to determine the percent of FFA in the oil, a process called titration is used. A waste cooking oil sample between 14 and 15 g was dissolved in 50 mL of ethanol/diethyl ether mixture (1:1 by volume). The sample is titrated with alcoholic KOH using phenolphthalein as indicator. This can also be confirmed by checking the pH of the mixture. A pH of about 9 signifies all of the FFA has been reacted.

*Reagents*

Solvent mixture (95% ethanol/diethyl ether, 1/1, v/v); 0.1 M KOH in ethanol accurately standardized with 0.1 M HCl (hydrochloric acid) (pure ethanol may be also used if aqueous samples are analyzed), 1 % phenolphthalein in 95% ethanol.

*Procedure*

10 g of filtered waste cooking oil was put in a glass flask and dissolve in at least 50 mL of the solvent mixture (if necessary by gentle heating). The oil sample was titrated, with shaking, with the KOH solution (in a 25 mL burette graduated in 0.1 mL) to the end point of the indicator (5 drops of indicator-phenolphthalein), making sure the pink colour persisting for at least 10 s. The acid value is calculated by the formula:

$56.1 \times N \times V / m$  where,  $V$  is the volume of KOH solution used in mL and  $N$  its exact normality of KOH,  $m$  is the mass in g of the sample.

### *Hydrolysis of waste cooking oil*

Waste cooking oil was hydrolyzed under optimized conditions (oil/buffer ratio: 2:1 (w/w), pH 8, 40 °C) in a 50 mL flask with constant agitation for the emulsification of oil and water<sup>14</sup>. The reaction was carried out at 40 °C for 8 h using 10 parts (by weight) of oil in 5 parts (by weight) of buffer (pH 8) and 3 parts (by weight) of TTP-pH tuned lipase. After 5 h of hydrolysis, the mixture was separated into two phases: an oil phase consisting of FFA, mono-, di-, and triglycerides along with a water phase containing glycerol by centrifugation at 5,000 rpm for 20 min at room temperature. The oil phase was then distilled to remove the residual water. After distillation, the oil phase containing the fatty acids was used directly as the feedstock for biodiesel production via acid catalysis.

### *Acid-catalyzed esterification*

The feedstock (hydrolysed waste cooking oil) and methanol were reacted in the presence of an acid (sulphuric, hydrochloric, nitric, phosphoric, or acetic acid) catalyst at 50 °C under constant agitation. After the reaction, methanol was removed by evaporation at 65 °C and the mixture was allowed to separate into an upper organic phase and a lower aqueous catalyst phase.

The effectiveness of various acid catalysts (sulphuric, hydrochloric, nitric, acetic, and phosphoric acids) as catalyst in the esterification of the feedstock was also investigated. Reactions were carried out at 50 °C for 60 min using a feedstock to methanol molar ratio of 1:20. The catalyst concentration was 5% (v/v).

Investigation was carried out to see the effects of the concentration of catalyst and reaction time on biodiesel conversion. A feedstock to methanol molar ratio of 1:15 was used. Experiments using four different concentrations (0.5, 1, 2.5 and 5%) of sulfuric acid and a reaction time of 60 min at 50 °C were performed. Amount of biodiesel produced was measured as mentioned above.

The effect of the effect of the free fatty acid content of the feedstock on biodiesel conversion was investigated. Feedstock with varying free fatty acid contents was used for acid-catalysed biodiesel production. The conversion of biodiesel was calculated for each reaction which contained from 50 to 90% free fatty acid. These feedstocks were produced by time controlled enhance lipase enzyme hydrolysis reaction. Reactions were carried out using a feedstock to methanol molar ratio of 1:15. The catalyst concentration (sulphuric acid) was 2.5% (v/v). Amount of biodiesel produced was measured after 30 min of reaction for each of the feedstock used.

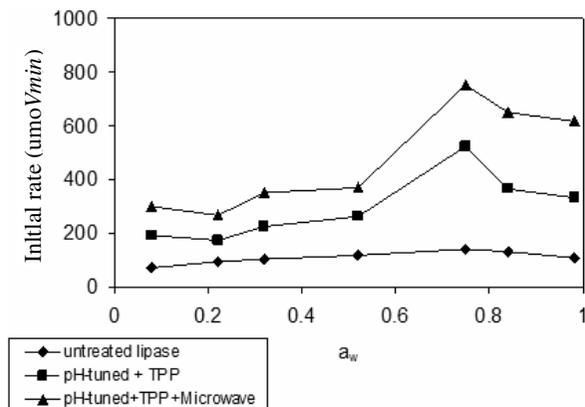
## **Results and Discussion**

In recent years, there has been growing interest in developing techniques for modification of lipases, in order to use them more economically and efficiently on large industrial scale. While enzymes are being increasingly used in organic solvents for a variety of applications, their low activity continues to be of major concern<sup>17</sup>. The role of lyophilisation for obtaining enzyme preparations for non-aqueous enzymology is rather recent. pH tuning and pH memory are observation for which clear rationalization is now available. The correct protonation state of side chains of amino acid residues of enzymes is important in non aqueous media as well. The dehydration step induces structural perturbation in the enzyme's secondary structure and these changes offers some advantages such as prevention of autolysis (in case of proteases) and increased thermo stability<sup>18</sup>. Hence pH tuning results in higher rates in organic solvents<sup>19,20</sup>. In this study

pH tuning of the lipase enzyme was done using 0.02 M tris-HCl buffer, pH 7.8. However instead of lyophilizing the enzyme, we used several short burst of microwave irradiation in cycles of 10 second each. After 15-20 cycle of ramp/cool treatment the enzyme solution was dry. This was known as the 'tuned enzyme'. Our experiments have shown that it takes only between 8-15 minutes to prepare the tuned enzyme. This drastically reduces the time taken to prepare tuned enzymes as compared with the current practice which requires overnight lyophilisation.

The two main parameters which are known to affect three phase partitioning are the amounts of ammonium sulphate and *t*-butanol added to the enzyme solution<sup>11</sup>. Previously it was known that 50% (w/v) ammonium sulphate saturation and 6 mL of *t*-butanol (added to 9.0 mg lipase in 3 mL of 0.02M tris-HCl buffer, pH 7.8) gave 3.2 times increase in the lipase activity in aqueous buffer. It was also shown that the TPP technique using these parameters did not separate significant amount of protein, as only 0.1 mg was left behind in the aqueous layer<sup>12</sup>. Hence in this study similar conditions with slight modification were used for the preparation of TPP-pH tuned lipase enzyme.

Figure 1 shows the effect of various hydration levels on the activity of untreated and TPP-pH tuned lipase. It is seen that with increasing hydration levels TPP-pH tuned enzyme showed higher reaction rates. Similar to pH-tuned enzyme, the water activity at the level of 0.79 in *n*-heptane gave the maximum initial rate even in the case of TPP-pH-tuned enzyme. The latter showed 3.9 times more activity than the untreated enzyme at this hydration level (Figure 1 and Table 1). The increase in activity of the TPP-pH tuned lipase is because of the increase in inherent catalytic power of the enzyme. TPP-pH tuned treatment can stabilize the structure and molecular flexibility of an enzyme that responsible for the enhancement of enzyme activity<sup>11</sup>. Thus once again, we observe a drastic reduction in time for dehydration process using microwave for the preparation of TPP-pH tuned enzyme.



**Figure 1.** Initial rates of *Candida rugosa* lipase, untreated, pH-tuned plus TPP-treated and pH-tuned plus TPP treated plus microwave irradiated, at various water activities.

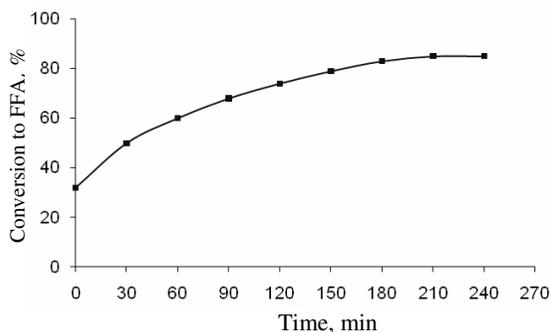
It was considered worthwhile to examine whether microwave irradiation had any effect on the pH memory. Application of microwave to promote reaction rates of chemical reactions has become fairly routine, but its application in enzyme-catalyzed reactions is relatively limited. Microwave-irradiation is relevant to non-aqueous enzymology because the enzymes in nearly anhydrous media are thermo-stable and in some cases it was reported

that keeping them at 100 °C for an extended period of time does not cause inactivation<sup>19</sup>. This is attributed to the fact that drying removes water molecules which were H-bonded to many surface residues. It results in these side chains interacting with each other and creating a rigid structure. When such powders are used in aqueous buffers, rehydration reverses such changes. On the other hand, when used in low water-containing organic solvents, rehydration is not possible and the structure remains highly thermo-stable. This means that one does not have to worry about thermal inactivation while using microwaves if the medium is nearly anhydrous. Microwave irradiation can be used in conjunction with other strategies for enhancing initial reaction rates. In this study (Table 1), microwave irradiation on TPP-pH tuned treated lipase resulted in an increase in lipase activity 5.4 times as compared to the rates observed in crude lipase. This study shows that combination of strategies may give a preparation with better catalytic properties.

**Table 1.** Enhancement of lipase activity with pH-tuning, three phase partitioning and microwave irradiation.

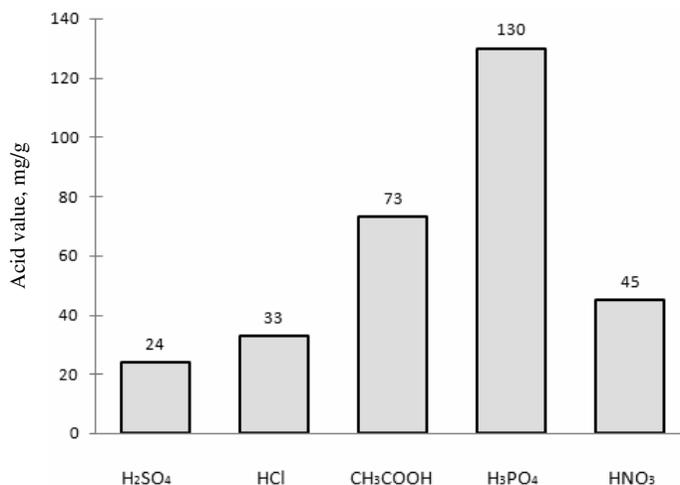
Lipase enzyme	Activity, in units	Increase in Activity, in units
Crude	840	1
PH- tuned	1995	2.4
pH-tuned plus TPP	3247	3.9
pH-tuned plus TPP and microwave irradiation	4515	5.4

The selection of feed stock with high acid value was done by the titration method. When the acid value of waste oil was about 70 mg KOH/g oil, it is reasonable to assume that the waste oil is composed of approximately 30%-40% free fatty acid, glyceride, monoglyceride, and diglyceride<sup>21</sup>. These samples were selected for the production of biodiesel in this study. Figure 2 displays the time course of waste cooking oil hydrolysis using the pH tuned plus TPP enhanced lipase enzyme. The reaction was carried out at 40 °C for 4 h using 10 parts (by weight) of oil in 5 parts (by weight) of buffer (pH 8) and 3 parts (by weight) of the pH tuned plus TPP enhanced lipase enzyme. From Figure 2, it can be seen that the conversion of FFA increased rapidly from 0 to 3 h. The percentage of free fatty acid produced was measured using the titration method. Acid value of 150 mg/g was used as an indicator that the free fatty acid content in the sample was approximately 85%<sup>21</sup>. After 3 hours, the conversion of FFA did not increase significantly, indicating that the feedstock for the acid-catalyzed biodiesel synthesis was easily obtained by the hydrolysis of the waste cooking oil using the lipase for duration of 3 h. To further improve the reaction in terms of the time required for the hydrolysis, microwave irradiation was used. Application of microwave to promote reaction rates of chemical reactions has become a fairly routine step. The reaction mixture above was subjected to microwave irradiation for 10 second at frequency of 2.45 GHz, a power output of about 100 W. The sample was then cooled. As mentioned previously, this ramp/cool cycle was repeated 15 times and then amount of free fatty acid produced was monitored by titration. After the 15<sup>th</sup> cycle of ramp/cool microwave irradiation the amount of free fatty acid produced was about 85% similar to the amount that was obtained after 3 h of reaction time using the non-microwave method. The feedstock obtained after 15<sup>th</sup> cycle of the microwave hydrolysis using the enhanced lipase enzyme which contained almost 85% free fatty acid was then subjected to acid catalyzed biodiesel production.



**Figure 2.** Time course of waste cooking oil hydrolysis catalyzed by pH-tuned plus TPP enhanced lipase enzyme. (The reaction was carried out using an oil/buffer ratio of 2:1 (w/w), pH 8 and at 40 °C).

The alkali-catalyzed transesterification of oils proceeds faster than the acid-catalyzed transesterification when refined oils (low acid value, high cost) are used, whereas unrefined oils or waste grease (high acid value, low cost) can be successfully transesterified by acid catalysis<sup>22</sup>. The deactivation of the alkali catalyst by the high FFA contents of the oil, leading to lower biodiesel yields, has been observed<sup>2,5</sup>. The effectiveness of various acid catalysts (sulphuric, hydrochloric, nitric, acetic, and phosphoric acids) (5%, v/v) in the esterification of the feedstock with a feedstock to methanol molar ratio of 1:20 was investigated and the results are shown in Figure 3.



**Figure 3.** Effect of different acid catalyst on biodiesel production.

Reactions were carried out at 50 °C for 1 h using a feedstock to methanol molar ratio of 1:20. The catalyst concentration was 5% (v/v). The initial acid value of the feedstock was 150 mg/g oil. The drop in acid value was used as a measure to calculate the percentage of conversion of feedstock to biodiesel.

Strong acids (sulphuric, hydrochloric, or nitric acid) exhibited higher catalytic activity as compared to weak acids (phosphoric or acetic acid). The initial acid value of the feed stock was 150 mg/g oil which gives about 85% free fatty acid content. The highest reduction

in the acid value was obtained in the presence of sulphuric acid which showed the acid value dropping from 150 mg/g to 24 mg/g after 1 hour of reaction at 50 °C. This could be inferred to a drop in free fatty acid content from 85% to 14%. This is about 83% conversion to biodiesel although no attempt was made in this study to quantify the content of the biodiesel produced by any chromatographic method. The acid dissociation indexes (pKa) for hydrochloric, sulphuric, nitric, phosphoric, and acetic acids are known to be 6.1, 3, 1.38, 2.1, and 4.8, respectively<sup>23</sup>. However, the results of this study revealed that the pKa alone is not a good index to screen a suitable acid as a catalyst for esterification. Goff *et al.*<sup>5</sup> reported similar findings on the catalytic ability of various acids for transesterification of soybean oil. Sulphuric acid was selected as the catalyst for further studies due to the higher catalytic effectiveness, lower cost and easy removal from biodiesel by neutralizing with alkali to form insoluble salt.

Effect of catalyst concentration in acid-catalyzed esterification may also be increased by the use of higher amounts of the catalyst. To explore this possibility, the effects of the concentration of catalyst on biodiesel conversion were investigated at 50 °C, and the results are shown in Table 2. A feedstock to methanol molar ratio of 1:15 was used. Four different concentrations (0.5, 1, 2.5 and 5%) of sulphuric acid and a reaction time of 15, 30, 45 and 60 min were used in the investigation. As shown in Table 2, using higher concentrations of the acid catalyst resulted in higher conversions of biodiesel. The conversion was almost similar after 60 min of reaction regardless of the acid catalyst concentration. The conversion of biodiesel increased from 65% to 80% when the catalyst concentration was increased from 0.5 to 5% within 15 min of reaction. The conversion to biodiesel with 5% sulphuric acid after 30 min of reaction was about 88%. The use of 2.5% or 5% sulphuric acid did not cause any significant difference in the esterification reaction rates. This indicates that esterification reaction reached equilibrium after 30 min of reaction under a feedstock to methanol molar ratio of 1:15 and 50 °C. These results show that a sulphuric acid concentration of 2.5% (v/v) and a shorter reaction time of 30 min were sufficient to yield up to 88% biodiesel conversion.

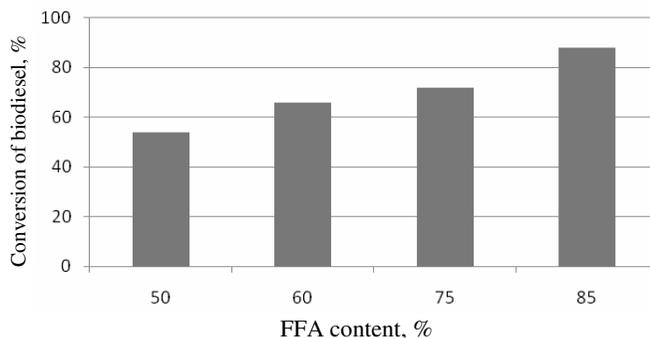
**Table 2.** The effect of acid catalyst (sulphuric acid) concentration on the esterification reaction.

Sulphuric acid concentration, %(v/v)	Acid value after 15 min reaction, mg/g	Acid value after 30 min reaction, mg/g	Acid value after 45 min reaction, mg/g	Acid value after 60 min reaction, mg/g
0.5	60	36	30	26
1	47	35	28	26
2.5	35	26	26	24
5	33	25	25	24

The reaction was carried out using a feedstock to methanol molar ratio of 1:15 at 50 °C. The feedstock contained 85% free fatty acid (acid value 150 mg/g oil)

Effect of free fatty acid (FFA) contents in the feedstock and its effect on the production of biodiesel were studied. From Figure 4, it can be seen that the conversion of biodiesel increased from 54 to 88% when the FFA content was increased from 50 to 85%, giving an indication that high FFA content was beneficial for biodiesel production. Rapid formation of biodiesel was observed in the 30 min of the reaction. With the formation of biodiesel, the reaction mixture was separated into two immiscible phases; a biodiesel phase (containing residual FFA and mono-, di-, triacylglyceride) and a methanol phase (containing acid catalyst). The conversion of biodiesel was calculated as percentage by weight of fatty acid methyl ester (FAME) formed divided by the weight of the feedstock initially taken for the

reaction. On the other hand, the conversion of biodiesel from mono-, di-, and triglycerides in these experiments was very low in the 30 min of reaction<sup>8</sup>. These results indicated that the higher biodiesel conversion obtained was due to the esterification of FFA and that FFA was more easily converted into biodiesel compared to triacylglycerides.



**Figure 4.** Effect of Free Fatty Acid content on biodiesel production.

Reactions were carried out at 50 °C for 30 min using a feedstock to methanol molar ratio of 1:15. The catalyst concentration was 2.5% (v/v). Fifty to 85% FFA was prepared by the enzymatic catalyst hydrolysis of waste oil using the pH-tuned plus TPP enhanced lipase enzyme. % of biodiesel produced was calculated as mentioned above

## Conclusions

This study has successfully develop an enzymatic/acid-catalyzed hybrid process for the production of biodiesel with a view to utilize non-edible and off quality waste cooking oil as feedstock. The waste oil was hydrolyzed in the presence of pH tuned plus TPP enhanced lipase enzyme using a binary method, and a higher degree of hydrolysis was achieved. The esterification of the feedstock by methanol at optimized conditions (50 °C; feedstock to methanol molar ratio of 1:15; 2.5% sulphuric acid) led to 88% conversion of biodiesel after 30 min. Problems linked to higher FFA contents can be overcome by using the enzymatic/acid-catalyzed hybrid process proposed in this study. Therefore, any unrefined oil which contains different levels of FFA can be used as a feedstock for biodiesel production. One of the main barriers to achieving higher biodiesel conversions is the presence of water, which is a by-product of the esterification. The transesterification of smaller esters is retarded in the presence of polar compounds<sup>24</sup>. The polar compounds will interfere in the reaction by competing for hydrogen ions, hindering the ability of these ions for catalysis, resulting in a reaction rate reduction. The water might play a similar role in the acid catalyzed synthesis of biodiesel. The water generated during esterification forms another phase which absorbs methanol and sulphuric acid<sup>5</sup> (Goff *et al.*, 2004). The acid catalysts preferentially bind to water, leading to a reversible type of catalyst deactivation. However, this process still showed an enhanced water tolerance as compared to the alkali-catalyzed process. This enzymatic/acid-catalyzed hybrid process would open up ways to utilize any unrefined and used oil with any percentage of free fatty acid as feedstock for biodiesel production.

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