Optimization of the Enzymatic Synthesis of Biodiesel from *Terminalia cattapa* L. Kernel Oil Using Response Surface Methodology

Dedy Suhendra¹*, Erin Ryantin Gunawan¹, Arista Dewi Nurita¹, Desy Komalasari² and Teguh Ardianto³

1 Chemistry Department, Faculty of Mathematics and Science, University of Mataram, Jl. Majapahit No. 62 Mataram 83125, INDONESIA
2 Mathematic Department, Faculty of Mathematics and Science, University of Mataram, Jl. Majapahit No. 62 Mataram 83125, INDONESIA
3 Physics Department, Faculty of Mathematics and Science, University of Mataram, Jl. Majapahit No. 62 Mataram 83125, INDONESIA

Abstract: The synthesis of biodiesel using *Terminalia cattapa* L. (local language: Ketapang) kernel oil and ethanol has been conducted. The effect of variable parameters of synthesis includes reaction time (3–7 h), temperature (35–60°C), amount of enzyme (0.1–0.3 g) and substrate molar ratio (ketapang kernel oil to ethanol, 1:1–1:3) were studied. Response surface methodology based on a five-level and four-variable central composite rotatable design were used to evaluate the interactive effects of the synthesis parameters on the percentage yield of biodiesels. The analysis of variance shows that the optimum conditions of the synthesis reaction were at 4.03 h of reaction time, 0.25 g of weight of enzyme, 45.04°C of temperature and 1.50 of substrate molar ratio. The actual experimental yield was 83.9% under optimum condition, which compared well to the maximum predicted value of 83.71%.

Key words: biodiesel, *Terminalia cattapa* L., response surface methodology

1 INTRODUCTION

Fossil fuel (petroleum) plays a very important role for the development of industry, transportation, agriculture and on almost all human activities. In other words, the modern world is very dependent on this fuel. However, the world is currently faced with two types of crisis generated by this fuel; the supply depletion and the environmental degradation resulting in global warming. One of the best ways to reduce the world’s dependence on petroleum is to develop renewable fuels, such as biodiesel. Biodiesel is defined as a mixture of mono alkyl esters of long chain fatty acids derived from renewable raw materials, such as vegetable oil or animal fat. The mono-alkyl ester of biodiesel has chain length of C14 – C22. Some of the vegetable oils used as raw material for biodiesel are palm, rapeseed, soybean and sunflower. However, these vegetable oils are edible oil. By converting edible oils into biodiesel, food resources are actually being converted into automotive fuels. Therefore, large-scale production of biodiesel from edible oils may bring global imbalance to the food supply and demand market. Eventually, with the implementation of biodiesel as a substitute fuel for petroleum-derived diesel oil, this may lead to the depletion of edible-oil supply worldwide. In order to overcome this phenomenon, this work have been conducted to produce biodiesel by using non-edible oils such as ketapang kernel oil.

In general, biodiesel produced through the transesterification of vegetable oil with short chain primary alcohol, mainly methanol or ethanol. Usually, this reaction catalyzed by metal ion in alkaline solution, such as: maltolate Ti (IV) and Zr (IV) complexes, Al₂O₃ and Na₂O, Nano La₂O₃, millimetric γ-Al₂O₃, etc. In addition to alkaline catalysts, acid catalysts also used in biodiesel synthesis, that is sulfuric acid. High catalytic activity shows by the alkaline catalysts, even at room temperature. However, when an alkaline catalyst is used, the content of free fatty acids (FFA) present in oils should be lower than 0.5 wt%. A larger amount of FFA content in the oil leads to the formation of unwanted soap in the product and difficulty in the separation of the biodiesel fuel from the glycerol formed during processing, which results in a decreased yield.

Recent studies show that biodiesel can be produced en-
zymatically by lipase-catalyzed transesterification which has become more attractive in biodiesel production since the glycerol can be recovered easily and the purification process for biodiesel is simple\textsuperscript{17, 18}. In addition, the use of lipase in biodiesel production tolerates the water content of oil and increases biodiesel yield by avoiding the soap formation. The classical method of optimization involves varying one parameter at a time and keeping the other constant. However, this method is inefficient as it fails to understand relationships between the variables(such as reaction time, temperature, molar ratio and amount of enzyme) and the response\textsuperscript{39}.

Response surface methodology is an effective statistical technique for the investigation of complex processes. The main idea of this method is to determine the influence of independent variables on the response, get a model of the relationship between independent variables and the response and get the process conditions that produce the best response. In addition, the advantage of this method is it does not require experimental data in large quantities\textsuperscript{39}.

In this work, enzymatic synthesis of biodiesel from Terminalia catappa L. kernel oil and ethanol was studied using Lipozyme TL, a commercial immobilized lipase. RSM comprising a five-level-four-factor central composite rotatable design was used to evaluate the interactive effects and to obtain the optimum conditions.

2 EXPERIMENTAL PROCEDURES

2.1 Materials

Immobilized lipase from Thermomyces lanuginosus (Lipozyme TL) was produced by Novo Nordisk (Denmark). Acetonitrile (HPLC grade) and fatty acid standards, e.g. oleic acid, palmitic acid, linoleic acid and stearic acid were obtained from Sigma Aldrich (USA). Ethanol and hexane were from T.J. Beaker (USA). Non-edible and non-commercial oil, that is ketapang kernel oil was extracted from ketapang fruit kernel. The oil purified by drain off into chromatography column and eluted using a mixture of hexane-diethyl ether (87: 13, v/v)\textsuperscript{21}. All the chemicals used were of analytical grade.

2.2 Experimental Design by RSM

The five-level-four-factor CCRD, a statistically-based experimental design approach, was employed. Based on the single-factor experiment results, reaction time, temperature, amount of enzyme, and substrate molar ratio are subjected to the further optimization. The experiment design contains 30 points, meanwhile, the fractional factorial design consisted of 16 factorial points, 8 axial points and 6 center points. The mathematical relationship relating the variables to the responses can be calculated by the quadratic polynomial equation\textsuperscript{22}:

\[ Y = b_0 + \sum_{i=1}^{4} b_i x_i + \sum_{i=1}^{4} b_{ij} x_i^2 + \sum_{i<j}^{4} b_{ij} x_i x_j \]

where \( Y \) is percentage of yield; \( b_0, b_i, b_{ij}, b_{ij} \) are constant coefficients and \( x \) is the encoded independent variables.

Design Expert (version 8.0.7, Stat-Ease Inc., Minneapolis, MN) software was adopted to perform the experimental design, data analysis, model fitting, and graph plotting.

2.3 Enzymatic Transesterification

The transesterification of Ketapang kernel oil with ethanol was studied. Different molar ratios of Ketapang kernel oil with ethanol were added to 10 mL n-hexane, followed by different amounts of enzyme. The mixture of Ketapang kernel oil, ethanol and Lipozyme TL were incubated in a horizontal water bath shaker (150 rpm) at different reaction temperature and reaction times.

2.4 HPLC Analysis

The percentage of conversion of Ketapang kernel oil into biodiesel was analyzed by using High Performance Liquid Chromatography (HPLC) Waters Breeze 1525 Preparative Gradient, USA. HPLC conditions used were acetonitrile-water mobile phase, column SGE C-18 ODS (250 mm, ID 4 mm, Frit 4/5 m), Waters 2489 UV / Visible detector and Waters 1525 binary HPLC pump. Another conditions are the wavelength of 213 nm, the composition of the eluent 90:10 (v/v) and the flow rate of 1 mL/min.

The concentration of biodiesel (esters) were calculated by equation: \( C_i = (A_i / A_{IS}) (C_{IS} D_{INS} / D_{INS}) \), where \( C \) is the amount of component \( x \) or internal standard, \( A \) is area for component \( x \) and \( D_{INS} \) is detector response factor for component \( x \) or internal standard \( (D_{INS} = A_x / C_x \) and \( D_{INS} = A_{IS} / C_{IS} \)). The percentage yield of biodiesel was calculated by equation: \( \text{percentage yield (％)} = (\text{mmol ester / mmol Ketapang kernel oil used}) \times 100 \).

3 RESULTS AND DISCUSSION

3.1 Model fitting

The results of CCRD experiments for studying the effects of four independent variables on biodiesel conversion are presented in Table 1 along with the predicted and observed values.

The predicted values obtained from model fitting technique using design expert software version 8.0.7.1 were seen to be sufficiently correlated to the observed values. Further analysis on the results showed that the reactions of ketapang kernel oil and ethanol were most suitably described with quadratic polynomial model. The second-order polynomial equation obtained was given below:
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Table 1 Central composite design quadratic polynomial model, experimental data, actual and predicted values.

| No. | Time (h) | Molar ratio (mmol) | Enzyme (g) | Temperature (°C) | Actual yield (%) | Prediction yield (%) |
|-----|----------|--------------------|------------|------------------|------------------|----------------------|
| 1   | 4        | 2.5                | 0.15       | 50               | 65.8             | 63.63                |
| 2   | 5        | 2                  | 0.2        | 45               | 75.92            | 73.49                |
| 3   | 4        | 1.5                | 0.25       | 40               | 80.37            | 80.50                |
| 4   | 4        | 2.5                | 0.25       | 50               | 72.58            | 71.23                |
| 5   | 7        | 2                  | 0.2        | 45               | 67.38            | 60.17                |
| 6   | 6        | 1.5                | 0.15       | 40               | 62.74            | 70.26                |
| 7   | 5        | 2                  | 0.2        | 45               | 79.23            | 73.49                |
| 8   | 4        | 1.5                | 0.25       | 50               | 82.43            | 82.59                |
| 9   | 5        | 2                  | 0.3        | 45               | 76.38            | 80.06                |
| 10  | 6        | 2.5                | 0.25       | 40               | 62.1             | 74.21                |
| 11  | 5        | 1                  | 0.2        | 45               | 79.29            | 74.20                |
| 12  | 3        | 2                  | 0.2        | 45               | 72.6             | 77.82                |
| 13  | 6        | 1.5                | 0.15       | 50               | 53.55            | 57.20                |
| 14  | 6        | 2.5                | 0.25       | 50               | 67.76            | 58.05                |
| 15  | 4        | 1.5                | 0.15       | 50               | 80.42            | 74.50                |
| 16  | 5        | 2                  | 0.2        | 35               | 78.29            | 72.23                |
| 17  | 5        | 2                  | 0.1        | 45               | 75.97            | 70.30                |
| 18  | 6        | 1.5                | 0.25       | 50               | 58.53            | 61.68                |
| 19  | 6        | 2.5                | 0.15       | 40               | 76.88            | 72.54                |
| 20  | 6        | 1.5                | 0.25       | 40               | 74.43            | 72.42                |
| 21  | 6        | 2.5                | 0.15       | 50               | 48.01            | 54.06                |
| 22  | 5        | 3                  | 0.2        | 45               | 62.01            | 65.12                |
| 23  | 5        | 2                  | 0.2        | 55               | 51.77            | 55.84                |
| 24  | 4        | 2.5                | 0.15       | 40               | 66.25            | 69.28                |
| 25  | 5        | 2                  | 0.2        | 45               | 71.17            | 73.49                |
| 26  | 4        | 2.5                | 0.25       | 40               | 82.4             | 74.56                |
| 27  | 5        | 2                  | 0.2        | 45               | 64.21            | 73.49                |
| 28  | 5        | 2                  | 0.2        | 45               | 67.85            | 73.49                |
| 29  | 5        | 2                  | 0.2        | 45               | 82.53            | 73.49                |
| 30  | 4        | 1.5                | 0.15       | 40               | 69.2             | 74.73                |

\[
\text{Yield} (\%) = 73.49 - 4.41A - 2.27B + 2.44C - 4.10D + 1.93AB - 0.90AC - 3.21AD - 0.12BC - 1.35BD + 0.58CD - 1.12A^2 - 0.96B^2 + 0.42C^2 - 2.36D^2
\]

where A is the time; B the molar ratio; C the amount of enzyme; D the temperature.

3.2 The Analysis of Variance (ANOVA)

Fisher’s statistical test using ANOVA was performed to evaluate the significance of the quadratic polynomial model. The ANOVA results, presented in Table 2, shows that the $F$ value of 1.51 for the lack of fit implies that it is not significantly relative to the pure experimental error, suggesting that the model correlates well with the experimental values. The non-significant lack of fit is also good as the primary objective was the model should fit the experimental data\(^{23}\). The $R^2$ value, 0.6216, showed that the model obtained was able to give a good estimate of response of the system in the range studied. Table 2 also shows that the variables, time, molar ratio and amount of enzyme are the most significant in the process. Meanwhile,
temperature had a less significant effect on this reaction. Similar results were shown by Gunawan et al.\textsuperscript{24} who determined the optimization of lipase-catalyzed transesterification for palm-based wax ester.

### 3.3 The Response Surface and Contour Plots Interpretation

#### 3.3.1 Interactive Effect of Reaction Time and Temperature

Three-dimension response surface and contour plots were made to investigate the relationship between different variables and response, in order to obtain the optimal transesterification conditions that would maximize the yield of biodiesel. Figure 1 shows the response surface plots as function of reaction time, temperature and their mutual interaction on biodiesel synthesis. This process was held at the fixed values of enzyme amount and substrate molar ratio in their center values. Figure 1 shows that the yields decrease with increase in reaction time. Figure 1 also shows that the yield increase slowly with increase in temperature reaction. The % yield increased on going from 40 to 45°C and thereafter starts to decrease. In this case, increasing the temperature increases yields in this endothermic reactions, as well as promotes collisions between enzyme and substrate molecules and results in enhancement of the reaction rate, but high reaction temperatures may result in enzyme denaturation\textsuperscript{25}. The maximum % yield obtained at 4.03 h of the reaction time and 45.04°C of the reaction temperature.

#### 3.3.2 Interactive Effect of Varying Amount of Enzyme and Substrate Molar Ratio

Figure 2 shows the response surface and contour plot of interactive effect between varying amount of enzyme and substrate molar ratio by fixing the reaction time and reaction temperature at their center points. The figure shows that increasing amount of enzyme will lead to an increased % yield. This may be due to that increasing of enzyme molecules can increase the number of catalytic active sites and hence more substrate molecules are converted into products. The result shows that the yield of biodiesel declined with the increase of amount of enzyme. This phenomenon may be due to that an excessive ethanol can exert substrate inhibition effect on lipase activity\textsuperscript{26}. However, at low substrate levels and low amount of enzyme, high conversion could be achieved (more than

### Table 2 ANOVA for the quadratic polynomial model.

| Source     | Sum of squares | Degree of freedom | Mean square | F-value | Prob > F |
|------------|----------------|-------------------|-------------|---------|----------|
| Model      | 1611.9         | 14                | 115.08      | 1.76    | 0.1446   |
| A-time     | 467.20         | 1                 | 467.20      | 7.15    | 0.0174   |
| B-molar ratio | 123.53       | 1                 | 123.53      | 1.89    | 0.1895   |
| C-enzyme   | 142.94         | 1                 | 142.94      | 2.19    | 0.1600   |
| D-temperature | 402.87        | 1                 | 402.87      | 6.16    | 0.0254   |
| AB         | 59.64          | 1                 | 59.64       | 0.91    | 0.3547   |
| AC         | 13.09          | 1                 | 13.09       | 0.20    | 0.6610   |
| AD         | 164.54         | 1                 | 164.54      | 2.52    | 0.1335   |
| BC         | 0.24           | 1                 | 0.24        | 3.6×10\textsuperscript{-3} | 0.9527   |
| BD         | 29.35          | 1                 | 29.35       | 0.45    | 0.5131   |
| CD         | 5.39           | 1                 | 5.39        | 0.082   | 0.7779   |
| A\textsuperscript{2} | 34.59       | 1                 | 34.59       | 0.53    | 0.4782   |
| B\textsuperscript{2} | 25.17       | 1                 | 25.17       | 0.39    | 0.5442   |
| C\textsuperscript{2} | 4.91          | 1                 | 4.91        | 0.075   | 0.7877   |
| D\textsuperscript{2} | 153.16      | 1                 | 153.16      | 2.34    | 0.1467   |
| Residual   | 980.77         | 15                | 65.38       |         |          |
| Lack of Fit| 736.89         | 10                | 73.69       | 1.51    | 0.3394   |
| Pure Error | 243.88         | 5                 | 48.78       |         |          |

| Std. dev.  | 8.09           | R-Square          | 0.6216      |
| Mean       | 70.27          | Adj. R-Squared    | 0.2684      |
| C.V.%      | 11.51          | Pred. R-Squared   | 0.7730      |
| PRESS      | 4595.68        | Adeq. Precision   | 4.989       |
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3.3.3 Interactive Effect of Temperature and Amount of Enzyme

As shown in Fig. 3A-B, the production of biodiesel increased with the temperature changing from 40 to 50°C, and a gradual decrease occurred over 45°C. This may be due to that temperature influences the reaction process by altering the collision frequency between enzyme and substrate, and influences the enzyme activity by changing the enzyme conformation. This is similar behavior with Fig. 1, increasing the temperature increases yields. However, any further increase in temperature above the optimum value might lead to enzyme inactivation.

3.4 Optimization of Reaction Conditions and Model Verification

According to the discussion above, it is possible to obtain a high yield of biodiesel through searching for the optimum point. Hence, five sets of predicted reaction conditions are given by the model, as shown in Table 3. The optimum conditions that giving the maximal biodiesel yield were temperature 45.04°C, reaction time 4.03 h, amount of enzyme 0.25 g, and molar ratio 1.50. Under these conditions, the actual conversion rate is up to 83.9%, which was in good accordance with the predicted value. Furthermore, this model was also validated with additional experiments under the selected random conditions while fixing the reaction time at the optimized value. Therefore, the predicted model was considered to be reliable and robust.
CONCLUSION

In this work, biodiesel was successfully synthesized by lipase-catalyzed transesterification of ketapang kernel oil with ethanol. The optimum reaction condition was studied by central composite rotatory design and response surface methodology. Comparison of predicted and experimental values revealed good correspondence between them, and it can be used to adequately describe the relationship between the factors and response. The most suitable combination of variables was 45.04°C, 4.03 h, 0.25 g and 1.50 for the reaction temperature, the reaction time, the amount of enzyme, and the substrate molar ratio, respectively. At these optimal conditions, the conversion yield reached 83.9%. This study may provide useful tools to develop economical and efficient processes for industrial production of biodiesel from ketapang kernel oil.

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