Enzymatic epoxidation of biodiesel optimized by response surface methodology

Hao Lu, Shangde Sun, Yanlan Bi* and Guolong Yang

Lipid Technology and Engineering, School of Food Science and Engineering, Henan University of Technology, Lianhua Road, Zhengzhou 450001, Henan Province, P. R. China.

Accepted 20 June, 2012

During the enzymatic epoxidation of biodiesel, stearic acid was selected as oxygen carrier. Enzyme screening and the load of stearic acid were investigated. The effects of four main reaction conditions including reaction time, temperature, enzyme load, and mole ratio of H$_2$O$_2$/C=C-bonds on the epoxy oxygen group content (EOC) of epoxidized biodiesel were analyzed. Response surface methodology (RSM) was employed to study and optimize the reaction variables in the enzymatic epoxidation of biodiesel. A second-order model satisfactorily fitted the data ($R^2 = 0.9893$), with non-significant lack of fit. A higher EOC ($6.40 \pm 0.20\%$) of epoxidized biodiesel was obtained at the optimum conditions of 55.4°C, 3.91% enzyme load (relative to the weight of biodiesel), 2.93:1 mole ratio of H$_2$O$_2$/C=C-bonds, and 7.3 h. The relationship between initial reaction rate and temperature was also established, and the activation energy (Ea) of the enzymatic epoxidation is 16.03 KJ/mol.

Key words: Epoxidation, biodiesel, novozym 435, stearic acid, response surface methodology.

INTRODUCTION

Epoxidized vegetable oils such as soybean oil, linseed oil, and rapeseed oil, can be used as biodegradable and non-toxic lubricants, plasticizers and stabilizers for the plastics of polyvinyl chloride (PVC), and rubber-like materials (Thames and Yu, 1999; Wu et al., 2000; Adhvaryu and Erhan, 2002; Okieimen, 2002; Xu et al., 2002; Hwang et al., 2003; Sharma et al., 2006). Epoxidation of unsaturated vegetable oils have been carried out on industrial scale by the Prileshajev-epoxidation, using either preformed or short chain peroxy acids, which need strong mineral acids, such as H$_2$SO$_4$ and H$_3$PO$_4$ (Gerbase et al., 2002; Petrovic et al., 2002), to catalyze peroxy acids formation in these processes. However, these chemical epoxidation can sometimes initiate undesirable oxirane ring-opening reactions and result in unsatisfactory selectivity.

Recently, to overcome these disadvantages, successful epoxidation of vegetable oils through enzymatic routes have been reported (Rüsch gen Klaas and Warwel, 1996, 1999; Piazza et al., 2001; Orellana-Coca et al., 2005a, b; Vlček and Petrović, 2006; Törnvall et al., 2007) but the above-cited reports mainly focused on the study of vegetable oils and fatty acids. Biodiesel are biodegradable and nontoxic renewable resource (Knothe et al., 2005; Narasimharao et al., 2007). These biodiesel can be epoxidized to produce epoxidized products (Campanella et al., 2008; Holser, 2008), which are potentially useful intermediates for further manufacture of commercially important materials, and can also be used directly as plasticizers and plastic stabilizers. However, these previous reports only focused on chemical epoxidation of biodiesel (Campanella et al., 2008). In our previous report, it is very interesting that the effects of different free fatty acids used as active oxygen carrier on the enzymatic epoxidation were different, and stearic acid showed good performance in converting biodiesel double bonds to oxirane groups, than traditional oxygen carriers, such as, acetic and formic acid (Lu et al., 2010).

*Corresponding author. E-mail: yanlanbi@hotmail.com. Tel: +86-371-67788916. Fax: +86-371-67788916.

Abbreviations: EOC, Epoxy oxygen group content; RSM, response surface methodology; Ea, activation energy.
However, no studies focused on the effects of reaction process and parameters on the enzymatic epoxidation of biodiesel.

The aim of the present work was to investigate the effect of stearic acid as active oxygen carrier on the epoxidation of biodiesel, and also study the relationships between the reaction parameters (reaction temperature, temperature, enzyme load, and mole ratio of H₂O₂/C=C-bonds) and the response (epoxy oxygen group content, EOC) to determine the optimum conditions using Box-Behnken design and response surface methodology (RSM). Enzyme screening and the effect of stearic acid load were also investigated.

MATERIALS AND METHODS

Soybean oil (iodine value = 133.0) was purchased from Henan Cereals Oils Sunshine Oil and Fat Company Limited (Henan, China), hydrogen peroxide (purity = 35%) was purchased from Luoyang Haohua Chemical Company Limited (Luoyang, China), stearic acid (purity > 99%; peroxide value = 0.69 mmol/kg) was purchased from Sigma-Aldrich Company (St. Louis, MO), biodiesel were prepared by esterification of soybean oil and methanol, catalyzed by alkali. Immobilized lipase Lipozyme RM IM (from Rhizomucor miehei, RML), lipozyme TL IM (from Thermomyces lanuginose, TLL), and Novozym 435 (from Candida antarctica B, CAL) were provided by Novozymes A/S ( Bagsvaerd, Denmark). All other reagents were of analytical grade.

Enzymatic epoxidation of biodiesel

Epoxidation reactions were carried out in 250 mL three-necked round-bottom flasks, and placed in a water bath. Biodiesel (10 g) was reacted with stearic acid (2.8 g) and immobilized lipase (0.3 g) in toluene (50 g) at 55°C, and 35% hydrogen peroxide (14 g) was added dropwise in first 10 min to the reaction mixture. At the end of the epoxidation, immobilized biocatalysts were removed by filtration. Prior to analysis, the samples were washed with distilled water, with trace water decomposed traces of unreacted peroxide removed with anhydrous sodium sulfate. The solvent and the remaining trace water were distilled off using rotary evaporator.

Experimental design

A three-level-four-factor Box-Behnken design was employed in this study. The variables and their levels selected for the epoxidation of biodiesel were reaction temperatures (45.0, 55.0 and 65.0°C), enzyme load (1.0, 3.0 and 5.0%, relative to the weight of biodiesel), mole ratio of H₂O₂/C=C-bonds (1:1, 2.5:1, 4:1), and reaction time (4.0, 10.0, 16.0 h). Table 1 shows the independent factors (X), levels, and experimental design using coded and uncoded parameters. All the experiments were performed in triplicate.

Analytical techniques

Epoxy oxygen group content (EOC) determination was carried out by the direct method with hydrobromic acid solution in acetic acid (Paquot, 1979). From the oxirane content, the percentage relative conversion to oxirane was determined using the following formula:

Relative conversion to oxirane (RCO) = \( \frac{\text{OOex}}{\text{OOth}} \times 100 \)  \hspace{1cm} (1)

Where, \( \text{OOex} \) is the content of oxirane oxygen experimentally determined and \( \text{OOth} \) is the theoretical maximum oxirane oxygen content in 100 g of biodiesel, determined using the following expression:

\[
\text{OOth} = \left( \frac{(\text{IV}_0 - 2\text{AI})}{(100 + (\text{IV}_0 - 2\text{AI}) \times A_0)} \right) \times 100
\]  \hspace{1cm} (2)

Where \( \text{AI} \) (126.9) and \( A_0 \) (16.0) are the atomic weights of iodine and oxygen, respectively and \( \text{IV}_0 \) is the initial iodine value of biodiesel.

Statistical analysis

Box-Behnken design for four independent reaction parameters (reaction temperature, enzyme load, mole ratio of H₂O₂/C=C-bonds, and reaction time) was used to obtain the combination of values that optimizes the response (EOC of epoxidized biodiesel) within the region of the three dimensional observation space, which allows one to design a minimum number of experimental runs. The model evaluates the effects of each independent variable on response. 3rd factor tests allow efficient estimation of a second order polynomial regression. The mathematical relationship relating reaction parameters to the response can be calculated by the quadratic polynomial equation:

\[
Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j
\]  \hspace{1cm} (3)

Where \( Y \) is one of the response (EOC); \( X_i \) and \( X_j \) represent the independent reaction parameters; \( \beta_0 \) is the constant; \( \beta_i \) is the linear term coefficient; \( \beta_{ij} \) is the quadratic term coefficient and \( \beta_{ij} \) is the cross term coefficient. The experiments were performed for analysis using Design expert. For the study, a total of 29 tests were necessary to estimate the coefficients.

RESULTS AND DISCUSSION

Enzyme screening

Novozym 435 (Candida antarctica B), Lipozyme RM IM (Rhizomucor miehei), and Lipozyme TL IM (Thermomyces lanuginosa), were screened for their abilities to epoxidize biodiesel. Lipozyme RM IM and Lipozyme TL IM had nearly no activity on epoxidation of biodiesel. Novozym 435 gave the highest epoxidation efficiency of biodiesel (EOC 6.30 ± 0.20%), and thus was selected for use in subsequent experiments.

Effects of stearic acid load

With the increase of stearic acid load up to 28% (relative to the weight of biodiesel), the maximum EOC of epoxidized biodiesel increased to 6.30 ± 0.20%; after that the maximum EOC of epoxidized biodiesel almost maintained the same level (Figure 1), while the EOC of epoxidized biodiesel in the absence of stearic acid was lower than 1% at 12 h. These results were attributed to the oxygen carrier of stearic acid and enough stearic acid necessary for the enzymatic epoxidation.
Effects of stearic acid load on the epoxidation of biodiesel. Epoxidation conditions were as follows: biodiesel (10 g), immobilized lipase (0.3 g), toluene (50 g), 55°C, 800 rpm, and 35% hydrogen peroxide (14 g). □ (stearic acid load 14%, relative to the weight of biodiesel); ○ (stearic acid load 21%); △ (stearic acid load 28%); ▽ (stearic acid load 35%).

**Figure 1.** Effects of stearic acid load on the epoxidation of biodiesel. Epoxidation conditions were as follows: biodiesel (10 g), immobilized lipase (0.3 g), toluene (50 g), 55°C, 800 rpm, and 35% hydrogen peroxide (14 g). □ (stearic acid load 14%, relative to the weight of biodiesel); ○ (stearic acid load 21%); △ (stearic acid load 28%); ▽ (stearic acid load 35%).

**Effect of reaction temperature**

Figure 2A shows that reaction temperature had a considerable influence on the EOC of epoxidized biodiesel. With the increase of reaction temperature, the EOC of epoxidized biodiesel reached the maximum (6.10 ± 0.20%) when the temperature raised from 35 to 55°C at 8 h but the EOC of epoxidized biodiesel declined after that (55°C), which may be ascribed to the enhancement of oxirane ring-opening side reaction. The initial reaction rates, defined as the initial oxirane ring formation per unit time ($V_0$, mol/L min), were calculated from the EOC-time profile. So, the Arrhenius law can be described as follows:

$$\ln V_0 = \ln A - \frac{E_a}{RT}$$  \hspace{1cm} (4)

Where, $A$ is the Arrhenius constant; $E_a$ is the activation energy; $R$ is the gas constant and $T$ is the absolute temperature (K). The value of $E_a$ obtained by means of the above-mentioned equation (4) is 16.03 KJ/mol (Figure 2B). Thus, the Arrhenius law equation about the initial reaction rate and the reaction temperature (35 to 55°C) can be written into equation (5):

$$\ln V_0 = 3.0572 - \frac{1928.4}{T}$$  \hspace{1cm} (5)

**Model fitting**

Modeling of variables and response was performed by RSM to predict the highest possible EOC of epoxidized biodiesel. The results obtained for the models were listed in Table 1. The greatest EOC of epoxidized biodiesel (6.45 ± 0.10%) was related with treatment [10 h, 55°C, 3.0% enzyme load (relative to the weight of biodiesel), 2.5:1 (mole ratio of H$_2$O$_2$/C=C-bonds)], and the lowest EOC of epoxidized biodiesel (1.68 ± 0.04%) with treatment (10 h, 65°C, 1.0%, 2.5:1), both among all the treatments tested. The data were analyzed employing a multiple regression technique to develop response surface models. Both a linear model and a second-order model were tested, using an F-test at the 95% confidence level. The following two second-order model satisfactorily explained the EOC of epoxidized biodiesel, with non-significant lack of fit (Table 2). $EOC_{biodiesel}$ is the predicted...
value for EOC of epoxidized biodiesel, and $X_1$, $X_2$, $X_3$ and $X_4$ are the coded variables as described in Table 1.

$$\text{EOC}_{\text{biodiesel}} (\%) = 6.35 + 8.33 \times 10^{-2} X_1 + 0.56 X_2 + 0.93 X_3 - 0.17 X_4 - 0.18 X_1^2 - 1.23 X_2^2 - 1.16 X_3^2 - 0.63 X_2 X_3 - 0.01 X_1 X_3 - 0.24 X_1 X_4 + 0.97 X_2 X_3 + 0.97 X_2 X_4 + 0.24 X_3 X_4$$

The relationship between reaction variables and response can be better understood by examining the planned series of 2-D contour plots (Figure 3) generated from the predicted model. Figure 3A shows the effect of reaction temperature, reaction time and their mutual interaction on the EOC of epoxidized biodiesel. The maximum EOC (>6.39%) of epoxidized biodiesel appeared in the temperature range of 50 to 55°C and time range of 9 to 14 h. The decrease of EOC of epoxidized biodiesel at higher temperatures (>60.0°C) could be attributable to the enhancement of oxirane ring-opening side reaction.

Figure 3B shows the effect of varying enzyme load and reaction time on the epoxidation of biodiesel. The effect of enzyme load was more significant than that of reaction time in the process. During the reaction time tested, maximum EOC could be achieved at higher enzyme loads (3.0 to 4.4%). Figure 3C shows the effect of varying substrate ratio (mole ratio of $\text{H}_2\text{O}_2$/C-C-bonds) and reaction time on the epoxidation of biodiesel. Middle substrate ratio (2.5:1) and middle reaction time (10 h) resulted in maximum EOC (6.40%). Figure 3D shows the effect of varying enzyme load and reaction temperature on the epoxidation of biodiesel. Higher enzyme loads (3.0 to 4.4%) and middle reaction temperatures (50 to 57°C) resulted in maximum EOC (~6.40%) of epoxidized biodiesel. This can be explained by the fact that enough enzymes are very important for enzymatic epoxidation.

Figure 3E shows the effect of varying mole ratio of $\text{H}_2\text{O}_2$/C-C-bonds and reaction temperature on the epoxidation of biodiesel. Lower substrate ratios and lower reaction temperatures resulted in higher EOC of epoxidized biodiesel. However, if substrate ratio was lower than 1.70, a low reaction temperature (<50°C) would not further increase the EOC of epoxidized biodiesel, which is the result of lack of enough $\text{H}_2\text{O}_2$ for the epoxidation of C=C bonds at lower temperatures. Figure 3F showed the effect of varying the enzyme load and substrate ratio on the epoxidation of biodiesel. The higher EOC (>6.30%) appeared at higher enzyme loads (3.20 to 4.25%) and middle substrate ratios (1.75 to 3.25).

**Attaining optimum conditions**

Besides the exhibition of the effects of the variables on the response, the contour plots also indicated several desirable combinations of the variables that gave higher EOC of epoxidized biodiesel, which can provide options when practical aspects is considered in real commercialization of the process. We suggest, according to optimization by the contour plots, the following set of conditions: reaction time 7.3 h, reaction temperature 55.4°C, enzyme load 3.91% (relative to the weight of biodiesel), and substrate ratio, 2.93:1 (mole ratio of $\text{H}_2\text{O}_2$/C-C-bonds). Under the suggested conditions, the EOC of epoxidized biodiesel is estimated to be 6.46%.

**Model verification**

Experiments were done at the predicted optimum
Table 1. Experimental design and results obtained and predicted from the process.

<table>
<thead>
<tr>
<th>No</th>
<th>Time</th>
<th>Temperature</th>
<th>Enzyme</th>
<th>Substrate ratio</th>
<th>EOC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$X_1$ (h)</td>
<td>$X_2$ (ºC)</td>
<td>$X_3$ (%)</td>
<td>$X_4$ (mol/mol)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0(10.0)</td>
<td>0(55.0)</td>
<td>-1(1.0)</td>
<td>-1(1:1)</td>
<td>4.10±0.12</td>
</tr>
<tr>
<td>2</td>
<td>1(16.0)</td>
<td>0(55.0)</td>
<td>1(5.0)</td>
<td>0(2.5:1)</td>
<td>5.81±0.09</td>
</tr>
<tr>
<td>3</td>
<td>0(10.0)</td>
<td>0(55.0)</td>
<td>0(3.0)</td>
<td>0(2.5:1)</td>
<td>6.40±0.06</td>
</tr>
<tr>
<td>4</td>
<td>0(10.0)</td>
<td>0(55.0)</td>
<td>1(5.0)</td>
<td>1(4:1)</td>
<td>5.60±0.10</td>
</tr>
<tr>
<td>5</td>
<td>0(10.0)</td>
<td>-1(45.0)</td>
<td>0(3.0)</td>
<td>-1(1:1)</td>
<td>5.98±0.07</td>
</tr>
<tr>
<td>6</td>
<td>-1(4.0)</td>
<td>0(55.0)</td>
<td>0(3.0)</td>
<td>-1(1:1)</td>
<td>5.42±0.08</td>
</tr>
<tr>
<td>7</td>
<td>1(16.0)</td>
<td>-1(45.0)</td>
<td>0(3.0)</td>
<td>0(2.5:1)</td>
<td>6.02±0.10</td>
</tr>
<tr>
<td>8</td>
<td>1(16.0)</td>
<td>0(55.0)</td>
<td>-1(1.0)</td>
<td>0(2.5:1)</td>
<td>3.80±0.06</td>
</tr>
<tr>
<td>9</td>
<td>1(16.0)</td>
<td>0(55.0)</td>
<td>0(3.0)</td>
<td>1(4:1)</td>
<td>6.51±0.06</td>
</tr>
<tr>
<td>10</td>
<td>0(10.0)</td>
<td>-1(45.0)</td>
<td>0(3.0)</td>
<td>1(4:1)</td>
<td>5.31±0.07</td>
</tr>
<tr>
<td>11</td>
<td>-1(4.0)</td>
<td>0(55.0)</td>
<td>0(3.0)</td>
<td>1(4:1)</td>
<td>6.55±0.08</td>
</tr>
<tr>
<td>12</td>
<td>-1(4.0)</td>
<td>0(55.0)</td>
<td>-1(1.0)</td>
<td>0(2.5:1)</td>
<td>3.95±0.09</td>
</tr>
<tr>
<td>13</td>
<td>0(10.0)</td>
<td>0(55.0)</td>
<td>0(3.0)</td>
<td>0(2.5:1)</td>
<td>6.45±0.10</td>
</tr>
<tr>
<td>14</td>
<td>0(10.0)</td>
<td>-1(45.0)</td>
<td>-1(1.0)</td>
<td>0(2.5:1)</td>
<td>4.67±0.03</td>
</tr>
<tr>
<td>15</td>
<td>0(10.0)</td>
<td>0(55.0)</td>
<td>0(3.0)</td>
<td>0(2.5:1)</td>
<td>6.35±0.09</td>
</tr>
<tr>
<td>16</td>
<td>0(10.0)</td>
<td>1(65.0)</td>
<td>-1(1.0)</td>
<td>0(2.5:1)</td>
<td>1.68±0.04</td>
</tr>
<tr>
<td>17</td>
<td>0(10.0)</td>
<td>0(55.0)</td>
<td>-1(1.0)</td>
<td>1(4:1)</td>
<td>5.26±0.11</td>
</tr>
<tr>
<td>18</td>
<td>0(10.0)</td>
<td>0(55.0)</td>
<td>1(5.0)</td>
<td>-1(1:1)</td>
<td>5.50±0.04</td>
</tr>
<tr>
<td>19</td>
<td>0(10.0)</td>
<td>0(55.0)</td>
<td>0(3.0)</td>
<td>0(2.5:1)</td>
<td>6.30±0.04</td>
</tr>
<tr>
<td>20</td>
<td>1(16.0)</td>
<td>0(55.0)</td>
<td>0(3.0)</td>
<td>1(4:1)</td>
<td>4.68±0.10</td>
</tr>
<tr>
<td>21</td>
<td>0(10.0)</td>
<td>1(65.0)</td>
<td>0(3.0)</td>
<td>-1(1:1)</td>
<td>6.12±0.12</td>
</tr>
<tr>
<td>22</td>
<td>0(10.0)</td>
<td>0(55.0)</td>
<td>0(3.0)</td>
<td>0(2.5:1)</td>
<td>6.24±0.13</td>
</tr>
<tr>
<td>23</td>
<td>1(16.0)</td>
<td>1(65.0)</td>
<td>0(3.0)</td>
<td>0(2.5:1)</td>
<td>3.98±0.07</td>
</tr>
<tr>
<td>24</td>
<td>-1(4.0)</td>
<td>-1(45.0)</td>
<td>0(3.0)</td>
<td>0(2.5:1)</td>
<td>5.33±0.06</td>
</tr>
<tr>
<td>25</td>
<td>-1(4.0)</td>
<td>0(55.0)</td>
<td>1(5.0)</td>
<td>0(2.5:1)</td>
<td>6.01±0.10</td>
</tr>
<tr>
<td>26</td>
<td>0(10.0)</td>
<td>1(65.0)</td>
<td>1(5.0)</td>
<td>0(2.5:1)</td>
<td>5.30±0.08</td>
</tr>
<tr>
<td>27</td>
<td>0(10.0)</td>
<td>-1(45.0)</td>
<td>1(5.0)</td>
<td>0(2.5:1)</td>
<td>4.40±0.07</td>
</tr>
<tr>
<td>28</td>
<td>0(10.0)</td>
<td>1(65.0)</td>
<td>0(3.0)</td>
<td>-1(1:1)</td>
<td>3.10±0.05</td>
</tr>
<tr>
<td>29</td>
<td>0(10.0)</td>
<td>1(65.0)</td>
<td>0(3.0)</td>
<td>1(4:1)</td>
<td>4.70±0.11</td>
</tr>
</tbody>
</table>

Numbers were run in random order, bEnzyme load (% relative to the weight of biodiesel), cMole ratio of H₂O₂/C=C-bonds, dNumbers in parentheses represent actual experimental amounts.

Table 2. Regression analysis of variance for response surface quadratic model (ANOVA).

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>Degree of freedom</th>
<th>Mean square</th>
<th>F value</th>
<th>P &gt; F&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>EOC (%)</td>
<td>39.36</td>
<td>14</td>
<td>2.81</td>
<td>92.51</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Model</td>
<td>39.36</td>
<td>14</td>
<td>2.81</td>
<td>92.51</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>0.43</td>
<td>14</td>
<td>0.03</td>
<td>6.770×10⁻³</td>
<td>0.0512</td>
</tr>
<tr>
<td>Lack of fit</td>
<td>0.40</td>
<td>10</td>
<td>0.04</td>
<td>5.88</td>
<td>0.0512</td>
</tr>
<tr>
<td>Pure Error</td>
<td>0.027</td>
<td>4</td>
<td>6.770×10⁻³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>39.79</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Coefficient of variation = 3.47%, $R^2 = 0.9893$

<sup>a</sup>P < 0.05 indicates statistical significance.

Conditions to validate the RSM model, and the EOC of epoxidized biodiesel obtained was 6.40 ± 0.20%. Good agreements between the observed and predicted value indicated the validation of the model.

**FT-IR spectra of epoxidized biodiesel**

The disappearance of double bonds and the formation of epoxy groups were monitored during the epoxidation...
Figure 3. (A) Response contour plot between reaction temperature and reaction time with 3% (w/w, relative to the weight of biodiesel) enzyme load and 2.5:1 mole ratio of H$_2$O$_2$/C=C-bonds; (B) Response contour plot between enzyme load and reaction time with 2.5:1 mole ratio of H$_2$O$_2$/C=C-bonds at 55°C; (C) Response contour plot between substrate ratio (mole ratio of H$_2$O$_2$/C=C-bonds) and reaction time with 3% (w/w) enzyme load at 55°C; (D) Response contour plot between enzyme load and reaction temperature with 2.5:1 (mole ratio of H$_2$O$_2$/C=C-bonds) for 10 h; (E) Response contour plot between substrate ratio (mole ratio of H$_2$O$_2$/C=C-bonds) and reaction temperature with 3% (w/w) enzyme load for 10 h; (F) Response contour plot between substrate ratio (mole ratio of H$_2$O$_2$/C=C-bonds) and enzyme load at 55°C for 10 h.

using an FT-IR spectrometer (Figure 4). The peak due to the presence of double bonds in the biodiesel appeared at 3009 cm$^{-1}$, while the formation of the epoxy group at the specified reaction time could be concluded with the presence of 825 cm$^{-1}$ peak. Vleck and Petrovic (2006) also reported the presence of epoxy groups at 822 to 833 cm$^{-1}$, which agrees well with this study.

**CONCLUSION**

The epoxidation of biodiesel was successfully achieved
using Novozym 435 from *Candida antarctica* B as a biocatalyst. Stearic acid selected as oxygen carrier can enhance the epoxidation efficiency. RSM was used to model and optimize the epoxidation. A higher EOC of epoxidized biodiesel (6.40 ± 0.20%) was obtained at the optimum conditions of 55.4°C, 3.91% enzyme load (relative to the weight of biodiesel), 2.93:1 mole ratio of \( \text{H}_2\text{O}_2/\text{C} = \text{C}-\text{bonds} \), and 7.3 h.

**ACKNOWLEDGEMENT**

The work was supported by the earmarked fund for Modern Agro-industry Technology Research System (nycytx-20-1-08).

**REFERENCES**


