Enzymatic Methanolysis of Castor Oil for the Synthesis of Methyl Ricinoleate in a Solvent-Free Medium

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Abstract Several lipases of commercial grade were screened to catalyze the methanolysis of castor oil, and an immobilized Candida antarctica (Novozym 435) had the highest activity among the lipases tested. To enhance the yield of methyl ricinoleate, several reaction parameters were optimized. The optimum temperature was 50°C, and the original water content of lipase was sufficient to maintain the activity of lipase, and additional water supplied inhibited the methanolysis of castor oil. Because the lipase was deactivated by methanol, the reaction was tested by three-step addition of 1 molar equivalent of methanol to the oil. However, the oil was not completely converted to its methyl esters. The final reaction mixture using 3 molar equivalents of methanol to the oil consisted of 70% methyl ricinoleate, 18% monoricinoleate, 11% diricinoleate, and trace triricinoleate at the equilibrium state. The yield of methyl ricinoleate was 97% at 6 molar ratio of methanol to the oil with 300 g of castor oil and 6 g of immobilized Candida antarctica at 50°C within 24 h.

Keywords: Biodiesel, Candida antarctica, castor oil, fatty acid methyl ester, Novozym 435

Castor oil has a triglyceride of various fatty acids. The unique characteristics of castor oil is the very high content (87–90%) of ricinoleic acid, structurally 18-carbon hydroxylated fatty acid with one double bond [1]. Castor oil is distinguished from other triglycerides, because of its high specific gravity, viscosity, and hydroxyl value. Another unique feature is its alcohol solubility: It is miscible with absolute ethanol in all proportions, and the esters of ricinoleic acid have been widely used for industrial applications, including polymers, surfactants, deodorants, and germicides [9, 11].

Methanolysis of castor oil is similar to the synthesis of methyl esters from vegetable oils as diesel substitute. Because of environmental concerns, many researchers conducted the methanolysis of oils for the production of diesel oil or paramedical product using chemical methods [3, 13, 18]. However, the conventional chemical methanolysis has several drawbacks: difficulty in glycerol recovery, need for catalyst removal, and excess energy consumption [13]. To overcome these problems, the enzymatic alcoholysis of oils has been studied instead of chemical alcoholysis for the production of biodiesel fuel [6, 16, 17]. However, in the enzymatic methanolysis, the conversion from oils to their fatty acid methyl ester was low because of inhibition of enzyme by the water-stripping effect of methanol [4, 8, 12, 13]. To minimize the enzyme deactivation by methanol, two methods in general have been applied: stepwise addition of methanol and addition of organic solvent. Shimada et al. [16] conducted the reaction using stepwise addition of methanol to avoid the lipase deactivation by methanol and found that 98.4% of the oil was converted to its methyl esters. Iso et al. [7] carried out the transesterification of oils using methanol in the presence of organic solvent, 1,4-dioxane. These approaches are based on the assumption that methanol is insoluble in oils. Because of the insolubility of methanol in oils, oil-soluble organic solvent or small amount of methanol was added to enhance the solubility of methanol in oils. Apart from other oils, the methanolysis of castor oil may show different patterns for other oils, because methanol is very soluble in castor oil.

In this study, an appropriate lipase was screened and the effects of different reaction conditions on the enzymatic methanolysis of castor oil in a solvent-free system have been investigated, especially the effect of methanol content.
MATERIALS AND METHODS

Materials
Immobilized Candida antarctica (Novozym 435) and Rhizomucor melei (Lipozym RM) were obtained from Novo Nordisk ( Bagsvaerd, Denmark). Lipase from Alcaligenes sp. (Lipase QL, Lipase PL), Achromobacter sp. (Lipase AL), and Candida rugosa (Lipase OF, Lipase MY) were donated by Meito Sangyo (Nagoya, Japan). Castor oil (with fatty acid compositions of 89.5% ricinoleic, 4.2% linoleic, 3% oleic, 1% palmitic, 1% stearic, and 1.3% other acids) was purchased from Sigma (St. Louis, MO, U.S.A.), and the saponification value was 181 mg KOH/g castor oil, which was used to calculate the molar weight of the oil. Methyl ricinoleate was also purchased from Sigma. Methanol, ethanol, and acetonitrile of HPLC grade were purchased from Merck (Darmstadt, Germany).

Methanolysis of Castor Oil Using Lipase
Typically, methanolysis reactions were carried out by mixing 3 g of castor oil and 60 mg of lipase (2 wt% to the castor oil) with indicated amount of methanol. The reaction mixtures were shaken at 300 rpm and 50°C for the time indicated. To investigate the deactivation of lipase during reuse, batch reactions were repeated using the used lipase and fresh substrate mixture.

The effect of time on the yield of methyl ricinoleate was tested in 500-ml glass bottles using one-, two-, and three-step methanolysis of castor oil. Six g of lipase was mixed with castor oil and methanol at 250 rpm and 50°C. The first reaction in the step reactions was performed in a mixture of 300 g of castor oil and 1, 1.5, 2, or 6 molar ratio of methanol to the total fatty acids of castor oil, respectively. For the two-step reaction, 1.5 molar equivalent of methanol to the oil was added after 24 h, and 1 molar equivalent of methanol to the oil was added every 24 h for the three-step reaction.

To confirm the reversibility of methanolysis of castor oil, the initial mixture of 300 g of castor oil and 78.6 ml of methanol was reacted with 6 g of C. antarctica at 50°C for 100 h. Remaining methanol was evaporated under reduced pressure followed by filtration to remove the lipase. Six g of fresh C. antarctica was added into the mixture, and the final mixture was incubated at 50°C by shaking. At a specified time, the reacted mixture was sampled and analyzed using high performance liquid chromatography (HPLC). All tests were carried out in duplicate.

Analysis
The sampled reaction mixtures were centrifuged to remove glycerol, and the supernatant was heated for 24 h at 80°C to remove methanol. The remaining methanol after reaction was calculated using the difference of sample weight before and after evaporation. Fifty mg of the final sample was dissolved in 2 ml of ethanol and analyzed by HPLC.

Methyl ricinoleate and castor oil derivatives were analyzed on a reverse phase HPLC column (Waters symmetry 4 C18, column, 4.6×250 mm) with refractometer detector (Waters 410). The mobile phase consisted of acetonitrile/ethanol (3:2, v/v), and the flow rate was 0.5 ml/min.

RESULTS AND DISCUSSION

Screening of Lipase
Lipases, which are produced by microbe from various sources and are characterized by their activity hydrolyzing triacylglycerols, have a number of potential applications in oleochemistry, detergents, paper, and food industry and in

Table 1. Screening of lipase for the methanolysis of castor oil.

<table>
<thead>
<tr>
<th>Lipase</th>
<th>Yield of methyl ricinoleate (%)</th>
<th>30°C</th>
<th>50°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Powder</td>
<td>Immobilized</td>
<td>Powder</td>
</tr>
<tr>
<td>C. antarctica</td>
<td>-</td>
<td>52.6</td>
<td>-</td>
</tr>
<tr>
<td>Alcaligenes sp.</td>
<td>60.8</td>
<td>23.0</td>
<td>62.2</td>
</tr>
<tr>
<td>Alcaligenes sp.</td>
<td>57.8</td>
<td>13.5</td>
<td>50.8</td>
</tr>
<tr>
<td>R. melei</td>
<td>-</td>
<td>31.0</td>
<td>-</td>
</tr>
<tr>
<td>Achromobacter sp.</td>
<td>17.0</td>
<td>5.1</td>
<td>6.6</td>
</tr>
<tr>
<td>C. rugosa</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The reaction was conducted in a mixture of 3 g (3.23 mmol) of castor oil, 0.786 ml (19.38 mmol) of methanol, and 30 mg of lipase.

*The yield of methyl ricinoleate was calculated by dividing the weight percentage obtained and the theoretical maximum yield of the methyl ricinoleate.

*Lipase PL.

1Lipase OF and Lipase MY.

Experiment was not conducted.

N.D.
organic synthesis [2, 4–10, 12–17, 19–21]. In this study, for the enzymatic synthesis of methyl ricinoleate from castor oil, seven commercial lipases were screened at 30°C and 50°C. Among the lipases tested, C. antarctica had the highest methanolyis activity of castor oil at 50°C (Table 1). Although Alcaligenes sp. lipases also had high methanolyis activities, their activities decreased sharply when immobilized types were applied. For reusability of lipase, an immobilized type of C. antarctica was selected for the subsequent experiments.

Effect of Water Content on Methanolyis of Castor Oil
Laane and Tramper [4] reported that the activity of enzyme in a non-aqueous medium was affected by water content. In this study, the effect of water content on enzymatic activity was tested by adding a small amount of water to the reaction mixture. As the amount of added water increased from 0 to 1 wt% of the total amount of reaction mixture, the yield of methyl ricinoleate decreased gradually, and the highest yield was observed when no water was added (Fig. 1). This indicated that the initial water content in the lipase preparation was enough to catalyze out the methanolyis of castor oil in a solvent-free system.

Effect of Temperature on Methanolyis of Castor Oil
The effect of temperature on methanolyis of castor oil with C. antarctica was investigated in the range from 30°C to 60°C. The yield of methyl ricinoleate increased from 55% to 70% as the temperature increased from 40°C to 50°C, and the highest yield was obtained at 60°C (Fig. 2). Because the yields of methyl ricinoleate at 50°C and 60°C were almost the same, the following experiments were performed at 50°C to minimize the evaporation of methanol.

Effect of Molar Ratio on Methanolyis of Castor Oil
The reaction was conducted with 3 g of castor oil and 0.393 ml (3 mol ratio) to 1.572 ml (12 mol ratio) of methanol and 60 mg of C. antarctica at 50°C for 5 h.
Effect of Methanol Content on Methanolysis of Castor Oil

Although highly polar solvents such as methanol are known to deactivate enzymes, *C. antarctica* showed relatively high methanolysis activity of castor oil. However, deactivation of *C. antarctica* was observed at a high methanol concentration, and the inhibitory effect of methanol on methanolysis of castor oil depended on the amount of *C. antarctica* (Fig. 3). The maximum yield of methyl ricinoleate was obtained with nearly 6 molar ratio of methanol to the oil. The highest yield reached up to 47%, 67%, and 76% with 1 wt%, 2 wt%, and 3 wt% of *C. antarctica* for 5 h, respectively.

As mentioned previously, lipase was deactivated by methanol, because of its insolubility in oil. Although methanol is soluble in castor oil, deactivation of the lipase was also observed when the lipase was repeatedly used in the methanolysis of castor oils, similar to other oils (Fig. 4). This result showed that the deactivation of lipase did not occur by the insolubility of methanol, but rather seems likely that the methanol remained after reaction deactivated the lipase. The triglyceride is gradually converted to diglyceride, monoglyceride, and glycerol. Since methanol was used as a reactant in these series of reactions, the lipase might have been deactivated by the methanol remaining, because these reactions were taking place not simultaneously, but stepwise.

To eliminate the problem due to methanol, Shimada *et al.* [16] tested the stepwise addition of methanol: Equi-

Fig. 4. Effect of molar ratio of methanol to castor oil, when lipase was reused.
The reaction was conducted with 3 g of castor oil, 1–6 molar ratio of methanol to castor oil, and 60 mg of *C. antarctica* at 30°C for 5 h. After the reaction, products were removed except the lipase. Then, the fresh substrate (3 g of castor oil and 1–6 molar ratio of methanol to castor oil) was added. The reaction was repeated 4 times.

molar amount of methanol to oil was periodically added into the mixture every 24 h, and through stepwise addition of methanol to oil, the lipase could be used for 100 days without significant decrease of its activity. To confirm the feasibility of stepwise addition of methanol in the methanolysis of castor oil, the time profile was investigated in various step reactions (Fig. 5). In the single-step reaction using 6 molar ratio of methanol to oil, the yield of methyl ricinoleate reached 97% after 24 h, while the yield with 3 molar ratio of methanol to the oil was 80% after 72 h. In the second and third methanol additions during the three-step reaction, the yield increased gradually for the first 6 h, and decreased slowly after 6 h. This result can most likely be explained that the methyl ricinoleate produced was reconverted to monoglycerides, diglycerides, and triglycerides, because the methanolysis of castor oil is a reversible reaction. In the first methanol addition during the three-step reaction, the yield of methyl ricinoleate reached to the theoretical maximum yield of 33% and stayed unchanged. This result most likely indicated that methyl ricinoleate was converted to monoglycerides and diglycerides, but not to triglyceride.

In order to verify the above assumption, a four-step reaction and a reverse reaction were tested (Fig. 6). During the initial 3–4 h period of methanolysis, the amounts of methyl ricinoleate, monoricinoleate, and diricinoleate increased sharply, and the rate of increase was then suddenly diminished and almost reached a plateau before the methanol addition. When 2 molar equivalent of methanol was added after 24 h, the amount of methyl ricinoleate reached 50% within 1 h and then decreased slowly. Contrary to methyl
ricinoleate, however, the amounts of monoricinoleate and diricinoleate gradually increased, suggesting that methyl ricinoleate maintained the equilibrium with monoricinoleate and diricinoleate, and that the equilibrium state was altered by the amount of remaining methanol in the reaction. When the amount of methyl ricinoleate decreased, monoricinoleate and diricinoleate increased, while the content of remaining methanol in the four-step reaction was not changed. In addition, the remaining methanol did not react with ricinoleates to synthesize methyl ricinoleate. When methanol was not present in the system, the reverse reaction was predominant.

(Fig. 7). Methyl ricinoleate in the absence of methanol was converted to monoricinoleate and diricinoleate to the extent of 10% and 5%, respectively.

In summary, methanol was found to play two different roles in this experiment, such as deactivation of the lipase and incrementation of product yield, which are contrary to each other. In the former case, an immobilized C. antarctica was deactivated, when more than 1 molar equivalent of methanol to castor oil was used. To reduce the deactivation of the lipase, the reaction was tested by three- or four-step addition of 1 molar equivalent of methanol to castor oil was used. Although the equilibrium was shifted to monoricinoleate, diricinoleate and unreacted methanol remained. The methanol that remained was solubilized in the solution, because of the specific characteristic of castor oil, which is alcohol soluble making the lipase deactivated. In the latter case, the castor oil was not fully converted to its methyl esters, because the final reaction mixture using 3 molar equivalents of methanol to the oil consisted of 70% methyl ricinoleate, 18% monoricinoleate, 11% diricinoleate, and trace amount of triricinoleate at the equilibrium state. Contrary to the above, other oils such as soybean oil and rapeseed oil were fully converted to their methyl ester by 3 molar equivalents of methanol [16, 17, 19]. In castor oil, the equilibrium partially favored the mono or diglycerides reactants. However, in other oils, the equilibrium was shifted to methyl esters, which are products. Therefore, to completely convert the castor oil to its corresponding methyl esters, more than 6 molar equivalents of methanol to the oil are necessary.
Acknowledgment

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REFERENCES