Lipase-Catalyzed Synthesis of Palm-Based Wax Esters


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Abstract: Wax esters are long chain esters that are derived from long chain fatty acids and long chain alcohols with chain lengths of 12 carbons or more. The compounds have many potential applications. The present work focuses on the synthesis of wax esters by alcoholysis of palm oil with oleyl alcohol using Lipozyme. The effects of various reaction parameters such as reaction time, temperature, amount of enzyme, molar ratio of substrates, various organic solvents and initial water activity (a_w) of the reaction system were investigated. The optimum condition to produce wax ester were respectively, incubation time, 5 h-7 h, temperature, 40°C-50°C, amount of enzyme, 1.5% (w/v) and molar ratio of oleyl alcohol to palm oil, 3:1. Hexane was the best solvent for this reaction. Analysis of the yield of the products at optimum condition showed that between 78-83% wax esters were produced.

Key words: palm oil, Lipozyme, enzymatic, alcoholysis, wax ester, oleyl alcohol

1 Introduction

The Southeast Asian countries dominate the export trade of palm oil, whereby in 2003, Malaysia and Indonesia exported 13,354,000 and 9,750,000 ton of oil palm respectively (1). Thus, research in the development of palm oil to higher value added products is of great importance to these countries. Palm oil consists of triglycerides, combination of glycerol and different fatty acids. Alcoholysis of triglycerides from fats and oil to produce wax esters is relatively simple process and moreover, the starting materials are cheap. Salis et al. (2) reported that lipase-catalyzed alcoholysis of sheep milk fat with cetyl alcohol is by far the fastest of the transesterification reactions.

Wax esters are important ingredients in the cosmetic formulations (cleansers, conditioners and moisturizers) (3), in pharmaceuticals (as an anti foaming agent in the production penicillin and a timed release in the production pharmaceutical tablet) (4) lubricants, plasticizers and polishes (5,6). This is due their unique property of having excellent wetting behavior but without the oily feeling. Wax esters can be extracted from animals and plant materials such as beeswax, sperm whale and jojoba oil. However, they are often either too scarce or expensive for commercial use and the main obstacles to large-scale use them are its availability (7,8).

Wax esters can be synthesized using chemical and enzymatic methods. The use of homogeneous chemical catalyst may lead to several problems such as corrosion of equipment, hazards of handling of the corrosive acids, high-energy consumption and degradation of esters (8,9), where the enzymatic synthesis offers mild reaction conditions and environmentally friendly process.

In this work, the synthesis of wax esters using palm oil and long chain alcohols catalyzed by lipases was carried out. The effects of various parameters on alcoholysis reactions were investigated.

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2 Experimental Procedures

Materials: Immobilized lipase from *Mucor miehei* (Lipozyme), and lipase from *Candida antarctica* (Novozyme) were purchased from Novo Nordisk (Denmark). XAD7 (a macroporous crosslinked polymer made from polyacrylic ester) immobilized lipase was produced in our laboratory (10). Lipase from *Candida rugosa* was obtained from Sigma Chemical (USA). Lipases from *Aspergillus niger* and *Rhizopus niveus* were obtained from Fluka Chemika (Switzerland). Palm oil (MW = 3 × average of saponification equivalent of palm oil) (11) was obtained from Southern Edible Oil Sdn. Bhd. (Malaysia). Fatty acids composition in Malaysian palm oil is 0.1-0.3% of lauric acid, 0.9-1.5% of myristic acid, 39.2-45.2% of palmitic acid, 3.7-5.1% of stearic acid, 37.5-44.1% of oleic acid and 8.7-12.5% of Linoleic acid (12). Oleyl alcohol was obtained from Fluka Chemika (Switzerland). Ester standards, oleyl laurate, oleyl myristate, oleyl palmitate, oleyl stearate, oleyl oleate, oleyl linoleate and methyl linoleate were obtained from Sigma Aldrich (USA). Hexane, ethyl acetate, chloroform, heptane, nonane and isooctane were obtained from J.T. Baker (USA). All other chemicals were of analytical grade.

Alcoholysis reaction: The reaction mixture consisted of palm oil (1 mmol), oleyl alcohol (3 mmol) and enzyme (1.15 mg protein), unless otherwise stated. Hexane was added to a total volume of 10 cm³. The reaction mixture was incubated in a horizontal shaker waterbath with a speed of 150 rpm at 40°C for 24 h. All samples were assayed in triplicate and the experiments were repeated twice. The control experiments were carried out without enzyme.

Identification of the reaction mixtures: TLC plates (Merck type DC-plastic folein Keisel gel 60 F₂₅₄) were used to detect the component in the reaction samples. The developing system used was hexane:dry ether (8.5 : 1.5 v/v). The presence of the wax esters, palm oil and oleyl alcohol were detected as brown spots when a visualizing reagent, iodine was used.

Analysis of the reaction mixtures: The reactions were analyzed by a gas chromatograph (Hitachi model G-3000, Tokyo, Japan), using an Rtx-65TG capillary column (30 m × 0.25 mm). Helium was used as the carrier gas at a flow rate 30 mL/min. The temperature was programmed at 2 min at 150°C, 20°C/min to 300°C and 10 min at 300°C. The product composition was quantitated by an internal standard method with methyl linoleate as the internal standard. The concentrations of esters were calculated by equation: \( C_x = \frac{(A_x/A_{IS}) \times (C_{IS} \times D_{RF_{x}})}{D_{RF_{IS}}} \), where C is the amount of component x or internal standard, A is area for component x or internal standard and \( D_{RF_{x}} \) is detector response factor for component x or internal standard. \( D_{RF_{IS}} \) is detector response factor for internal standard. The percentage yield of ester was calculated by equation:

\[ \text{Percentage yield} = \frac{(\text{mmol ester/mmol palm oil used}) \times 100\%}{100\%}. \]

Screening of enzyme: The reaction mixture was incubated using various lipases. Amount of lipase used was based on the same amount of protein (1.15 mg protein). The lipases used were: Immobilized lipases; Lipozyme (0.145 g), Novozyme (0.150 g) and XAD7-immobilized lipase (0.130 g), free lipases; *Rhizopus niveus* lipase (0.070 g), *Candida rugosa* lipase (0.210 g), *Aspergillus niger* lipase (0.100 g). The percentage yield was determined as described earlier.

Effect of time: The effect of time on the alcoholysis reaction was investigated by varying the reaction time (0, 1, 2, 3, 4, 5, 7, 10, 15, 18, 21 and 24 h). The percentage yield was determined as described earlier.

Effect of temperature: The reaction mixture was incubated at various reaction temperatures (30, 40, 50, 60 and 70°C). The percentage yield was determined as described earlier.

Effect of amount of enzyme: The effect of increasing the amount of enzyme (Lipozyme) used (0.2, 0.5, 1.0, 1.5 and 2.0 % [w/g/vol(cm³)]) was studied. The percentage yield was determined as described earlier.

Effect of molar ratio of substrate: The reaction mixture was incubated with different molar ratio of substrates, \( n \) mmol oleyl alcohol/1 mmol palm oil. \( (n = 1, 2, 3, 4, 5, 7 \text{ and } 10 \text{ mmol}) \). The percentage yield was determined as described earlier.

Effect of various organic solvents: The effect of various organic solvents on the alcoholysis reaction was studied. The solvents used were acetonitrile (log P = 0.33), ethyl acetate (log P = 0.68), chloroform (log P = 2.00), hexane (log P = 3.50), heptane (log P = 4.00), isooctane (log P = 4.50) and nonane (log P = 5.00). The percentage yield was determined as described earlier.

Effect of initial water activity (aₙ): The effect of initial water activity (aₙ) on the alcoholysis reaction was studied. Lipozyme and the reactants were pre-equilibrated with the vapor of saturated salt solutions with different \( a_w \) values at temperature room in separate containers.
overnight (at least 16 h). The salts used were LiCl (0.11), MgCl$_2$.6H$_2$O (0.33), Mg(NO$_3$)$_2$.6H$_2$O (0.53), NaCl (0.75), KCl (0.86), KNO$_3$(0.9). The percentage yield was determined as described earlier.

3 Results and Discussion

Analysis of Products: The products were identified using gas chromatography by comparing the esters with the known standard. Quantitative analysis of the product was carried out by using methyl linoleate as internal standard which has retention time at 6.313 min. The standard esters used were oleyl laurate, oleyl myristate, oleyl palmitate, oleyl oleate and oleyl laurate. These esters have retention times at 10.263, 11.246, 12.576, 14.460, 14.710, 15.136 min, respectively.

Screening of Enzymes: Lipases from different sources were screened for alcoholysis reaction of palm oil and oleyl alcohol for 5 h. The percentage yields of six commercial lipases tested for the synthesis of wax esters is shown in Fig. 1. Lipozyme exhibited highest percentage yield at 75 %. Immobilized lipases, Novozyme and XAD-7 lipase also showed high percentage yields of alcoholysis. This finding was in agreement with the work of Athawale et al. (13) who reported that Lipozyme was the best lipase for the transesterification of soybean and linseed oils. Generally, Lipozyme is very active on long chain fatty acid (8). Other lipases showed only low percentage yield (less than 30%). Decagny et al. (14) reported that lipases from C. rugosa, R. javanicus and R. niveus were not efficient in producing wax ester (stearyl oleate).

Effect of reaction time: The time course of the enzymatic alcoholysis is presented in Fig. 2. The percentage yield increased with increasing reaction time. Lipozyme gave highest percentage yield within a reaction period of 5-7h. As expected oleyl palmitate gave the highest percentage yield (35%) compared to other wax esters. After 7h, the percentage yield was relatively constant. This may be due to the reactions has achieved the equilibrium state whereby the rate of forward reaction was equal to the rate of backward reaction. In the alcoholysis reaction between palm oil and oleyl alcohol, the products are not only wax esters but also glycerol. Glycerol will be accumulated and this may inhibit the reaction by limiting the interaction of the substrate and the enzyme (14,15). Previous work reported by Choo.

Fig. 1 Screening of Lipase. The reaction mixture consisted of palm oil (1 mmol), oleyl alcohol (3 mmol), hexane (to a total volume of 10 cm$^3$) and enzyme at 40°C and 150 rpm for 24 h. The lipases used were Lipozyme, Novozyme, XAD7-immobilized lipase, Rhizopus niveus lipase, Candida rugosa lipase, Aspergillus niger lipase. O. laurate : oleyl laurate, o. myristate : Oleyl myristate, O. palmitate : oleyl palmitate, O. stearate : oleyl stearate, O. oleate : oleyl oleate : O. linoleate : oleyl linoleate.
Effect of temperature: The percentage yield of wax ester was increased with increasing temperature from 30 to 50°C (Fig. 3). Similar trends were observed for all esters. Oleyl laurate gave the lowest percentage yield at 0.57%. This could be due to the low activation energy of the enzymatic reactions (17). The percentage yield of wax ester was decreased at temperature 60-70°C. This may be due to the thermal deactivation of the enzyme which occurs when the temperature was higher than 50°C (18). Similar result was reported by Mat Hadzir et al. (15) and Stevenson et al. (19).

Effect of amount of lipase: Fig. 4 depicts the result of using different amount of lipase. The percentage yield of wax esters was increased as the amount of enzyme was increased to 1.5%. The highest yields were found at 33.3 and 24.6% for oleyl palmitate and oleyl oleate, respectively. This behavior of leveling off alcoholysis at higher enzyme amount has also been reported by Mat Hadzir et al. (15). This can be explained by considering that the active sites of the enzyme molecules present in excess would not be exposed to the substrates and remain inside the bulk of enzyme particles without contributing significantly to the reaction (20).

Effect of molar ratio: The effect of oleyl alcohol to palm oil molar ratio on the alcoholysis (Fig. 5) indicated competitive nature of oleyl alcohol and fatty acid (in palm oil) binding. The molar ratio at 3:1 (oley alcohol: palm oil) produced highest percentage yield of oleyl palmitate (35.7%). A similar trend was observed for oleyl oleate, -linoleate, -stearate, -myristate and -laurate. This is similar with stoichiometric mixtures of reaction of palm oil and oleyl alcohol. Steinke et al.
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(21) reported that lipase-catalyzed esterification of stoichiometric mixtures of long-chain and very long-chain fatty acids with the corresponding mixture of alcohols gives quantitative yields of wax esters. The decreased in percentage yield of wax esters at higher oleyl alcohol concentration (4:1, 5:1, 6:1 and 7:1) may reflect the ability of the excess oleyl alcohol to distort the essential water layer that stabilizes the immobilized enzyme (22), which could inhibit the activity of enzyme.

Effect of organic solvents: The polarity of the various solvents in terms of their log-P values played crucial role in the course of the alcoholysis reaction (Fig. 6). Generally, the percentage yield increases with the increase in log-P value of the solvents. In this case, hexane (log P = 3.5) was found to be the best solvent to synthesize wax ester with percentage yield of 37.8% of oleyl palmitate. This finding was in agreement with Laane et al. (23) and Rahman et al. (24). This could be due to the fact that when the reaction medium is changed from hydrophilic (log P < 3) to hydrophobic (log P > 3) organic solvents, the overall efficiency of the enzyme changes (8). Basri et al. (10) explained that lipases function better in the more hydrophobic solvent. However, the solvents having a log P between 2 and 4

![Fig. 5](image1.png)  
**Fig. 5** Effect of Molar Ratio of Substrate (oleyl alcohol, n mmol/palm oil, 1 mmol). The reaction mixture consisted of palm oil (1 mmol), oleyl alcohol (3 mmol), hexane (to a total volume of 10 cm³) and Lipozyme (1.5%) at 50°C and 150 rpm for 5 h. O. laurate ; oleyl laurate, O. myristate ; oleyl myristate, O. palmitate ; oleyl palmitate, O. stearate ; oleyl stearate, O. oleate ; oleyl oleate, O. linoleate ; oleyl linoleate.

![Fig. 6](image2.png)  
**Fig. 6** Effect of Various Organic Solvents. The reaction mixture consisted of palm oil (1 mmol), oleyl alcohol (3 mmol), solvents (to a total volume of 10 mL) and Lipozyme (1.5%) at 50°C and 150 rpm for 5 h. The solvents used were acetonitrile (log P = 0.33), ethyl acetate (log P = 0.68), chloroform (log P = 2), hexane (log P = 3.5), heptane (log P = 4.0), isooctane (log P = 4.5) and nonane (log P = 5.0). O. laurate : Oleyl laurate, O. myristate : Oleyl myristate, O. palmitate : Oleyl palmitate, O. stearate : Oleyl stearate, O. oleate : Oleyl oleate, O. linoleate : Oleyl linoleate.
are weak water distorters and affect biological activity to an extent but rather unpredictable (23).

**Effect of initial water activity (aw):** The influence of water activity on the synthesis of wax esters by alcoholysis using Lipozyme is shown in Fig. 7. Chowdary et al. (25) suggested that water activity is strongly influenced by the hydration level of the enzyme and in turn affected the transesterification activity. However, in this particular alcoholysis reaction, this trend was not followed. In this study, the percentage yield of wax esters was not very much affected by changing the aw of the reaction mixture.

![Fig. 7](image)

**Fig. 7** Effect of Initial Water Activity (aw). The reaction mixture consisted of palm oil (1 mmol), oleyl alcohol (3 mmol), hexane (to a total volume of 10 mL) and Lipozyme (1.5%) at 50°C and 150 rpm for 5 h. The salts used were LiCl (0.11), MgCl₂.6H₂O (0.33), Mg(NO₃)₂.6H₂O (0.53), NaCl (0.75), KCl (0.86), KNO₃(0.90). O. laurate : oleyl laurate, O. myristate : oleyl myristate, O. palmitate : oleyl palmitate, O. stearate : oleyl stearate, O. oleate : oleyl oleate, O. linoleate : oleyl linoleate.

**Table 1** Synthesis of Palm-Based Wax Esters at Optimum Condition.

<table>
<thead>
<tr>
<th>Wax Esters</th>
<th>Carbon Number</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleyl Laurate</td>
<td>C 12:0</td>
<td>0.6-0.7</td>
</tr>
<tr>
<td>Oleyl Myristate</td>
<td>C 14:0</td>
<td>4.1-4.4</td>
</tr>
<tr>
<td>Oleyl Palmitate</td>
<td>C 16:0</td>
<td>33.3-38.0</td>
</tr>
<tr>
<td>Oleyl Stearate</td>
<td>C 18:0</td>
<td>4.0-4.1</td>
</tr>
<tr>
<td>Oleyl Oleate</td>
<td>C 18:1</td>
<td>26.6-29.9</td>
</tr>
<tr>
<td>Oleyl Linoleate</td>
<td>C 18:2</td>
<td>6-6.1</td>
</tr>
</tbody>
</table>

Alcoholysis reaction at optimum condition: The alcoholysis reaction was carried out at optimum condition (incubation period of 5 h, temperature at 50°C, amount of Lipozyme of 0.15 gram, molar ratio of substrates 1:3 (palm oil/oleyl alcohol), hexane as organic solvent). The percentage yield of wax esters obtained was 78.21-83.25%. The percentage yield of the different wax esters is shown in Table. 1. Generally, the composition of the wax esters produced coincides with the composition fatty acids in palm oil.

**4 Conclusion**

This work suggests that wax esters can be synthesized from palm oil and oleyl alcohol, by Lipozyme with high percentage of yield. Application of this process will produce high value-added product derivatives of palm oil.

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**References**

4. S. KLINE and French International Co, Improvements in or Relating to Pharmaceutical Tablet or Pellet and Method of Preparing the Same, GB 747914 (1956).
9. T. KNOX and K.R. CLIFFE, Synthesis of Long-chain Esters in a
Lipase-Catalyzed Synthesis of Palm-Based Wax Esters


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