

## Synthesis of Wax Esters by Lipase-catalyzed Esterification with Immobilized Lipase from *Candida* sp. 99-125\*

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**Abstract** Wax esters were synthesized in a solvent free system catalyzed by immobilized lipase from *Candida* sp. 99-125, with oleic acid and cetyl alcohol. The effects of substrate molar ratio, lipase dosage and water removal were investigated in a 50 ml flask incubated in a thermostatic cultivation cabinet. The optimized conditions were: temperature 40 °C, shaking at 170 r·min<sup>-1</sup>, acid/alcohol molar ratio 1 : 0.9, lipase dosage in 10% (by mass) of oleic acid, and open reaction for water removal. As a result, the conversion rate reached 98% for reaction of 8 h. The volume of reactor was scaled up to 1 L three-neck flask. The optimized parameters were: 200 r·min<sup>-1</sup> agitation speed, 2.5% (by mass) lipase dosage, others were the same as the parameters described above. The conversion rate reached 95% for reaction of 24 h. The lipase retained 46% conversion rate after reuse for 6, 7 batches. The products were purified by removing remained cetyl alcohol and fatty acids with ethanol and saturated sodium carbonate solution, respectively. The purity of the wax ester, cetyl oleate, was 96%. The physical and chemical properties of cetyl oleate were tested and compared with those of jojoba oil. The results show that the product cetyl oleate has great potential to use as the substitute of natural jojoba oil.

**Keywords** solvent free system, *Candida* sp. 99-125, lipase, wax ester

### 1 INTRODUCTION

Wax esters are long-chain esters derived from fatty acids and alcohols, both with chain lengths of twelve carbons or more [1]. They can be extracted from animal and plant materials such as beeswax, spermaceti oil and jojoba oil, and are widely used in lubricant, pharmaceutical, cosmetic and plasticizer industries [2]. Wax esters can be divided into saturated wax esters and unsaturated wax esters according to their chemical structures. Spermaceti oil and jojoba oil are the main sources of unsaturated wax esters [3], but sperm whale is listed as an endangered species in 1970 [4]. Jojoba oil is similar to spermaceti oil in some properties and can be used as a substitute, but the growth of jojoba plants is restricted to desert environment and its production cost is quite high [5, 6]. As a result, the natural unsaturated wax esters are too scarce and expensive for commercial use.

The synthesis of wax esters has been extensively investigated for many years, with the primary goal to use them as a substitute for spermaceti oil or jojoba oil. Chemical-catalyzed method has many disadvantages, such as corrosive acids required, hazards in handling corrosive chemicals, high energy consumption and degradation of synthesized esters [7, 8]. Lipase-catalyzed processes have attracted attention because of their mild reaction condition and environmental friendship. Owing to the mild reaction conditions, it is possible to obtain products that are difficult to be synthesized by

conventional chemical-catalyzed methods. Salis *et al.* synthesized wax esters from heavy fractions of sheep milk fat and cetyl alcohol in *n*-hexane, using immobilized lipase Lipozyme RMIM and Novozym 435 as catalyst [9]. Hadzir *et al.* synthesized wax esters by alcoholysis reaction between triolein and oleyl alcohol [10]. Gunawan *et al.* synthesized palm-based wax esters from palm oil and oleyl alcohol by Lipozyme IM [11]. Decagny *et al.* studied alcoholysis between triolein and stearyl alcohol to produce wax esters [12]. However, those studies are limited to laboratory scale for the expensive cost of production.

The energy saving and environmental friendship process to produce wax esters has been studied for years. Immobilized lipase from *Candida* sp. 99-125 has been made in the lab and used in enzymatic production of wax esters by esterification and transesterification, with palmitic acid, rapeseed oil, soybean oil and cetyl alcohol as substrates [13–17]. The processes have some problems, *e.g.*, the substrates and products are solidified easily, the process in packed-bed reactor is complicated and energy-intensive, and the solvents used in the process are volatile and potentially hazardous.

The present paper is related to the enzymatic synthesis of wax ester, cetyl oleate, from unsaturated fatty acids and cetyl alcohol with immobilized lipase from *Candida* sp. 99-125, in which the reaction is carried out in a solvent free system. Synthesis conditions, such as molar ratio of substrates, amount of lipase and water removal, are optimized. Then the reaction is

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scaled up to 1 L three-neck flask for further industrialization. The product is purified by removing remained free cetyl alcohol and fatty acids. In the end, the physical and chemical properties of cetyl oleate are examined and compared with those of jojoba oil.

## 2 MATERIALS AND METHODS

### 2.1 Materials

Oleic acid (the main fatty acids are oleic acid 44.71% and linoleic acid 30.37%), cetyl alcohol (98%) and all the other reagents such as ethanol, *n*-hexane, and sodium carbonate were purchased from Beijing Chemical Factory, Beijing, China and were of analytical grade. The lipase from *Candida* sp. 99-125 was immobilized on textile membrane [18–20] and was purchased from Beijing CTA New Century Biotechnology Co. Ltd., Beijing, China. The jojoba oil (main fatty acids: 11-eicosenoic acid, 64.4%; 2-docosenoic acid, 30.2%; oleic acid, 1.4%) was bought from Beijing Grandsun Bio-chemistry Co. Ltd., Beijing, China.

### 2.2 Optimization of esterification conditions

The reaction was carried out in a 50 ml flask incubated in a thermostatic cultivation cabinet at 40 °C and shaking at 170 r·min<sup>-1</sup>. Certain amounts of oleic acid and cetyl alcohol were put into the flask and dissolved by preheating. The immobilized lipase with activity of 8000 U·g<sup>-1</sup> [21] was added to the reaction system and the reaction started. Reactions were carried out in the flask with stopper or without stopper, and the reaction system with stopper was called close system, while the flask without stopper was named open system, allowing water to evaporate from the system. The reaction conditions involved several parameters, such as the amount of enzyme and molar ratio of substrates, but only one parameter was varied in the optimization study. At pre-determined time, samples were taken and measured by titration of remaining fatty acids with alkaline solution. Or 10 µl of sample was taken and dissolved in 1 ml *n*-hexane for gas chromatography (GC) analysis. All the experiments were replicated at least three times, and the results presented were the mean values for the replicated data.

### 2.3 Scale up and lipase stability

The volume of reactor was scaled up to 1 L three-neck flask based on the optimized parameters obtained in the 50 ml flask. The agitation speed was set to 200 r·min<sup>-1</sup> according to the previous results in the laboratory [22]. The lipase dosage and operation time were optimized, with the other parameters unchanged. Lipase stability was investigated in 1 L three-neck flask. The reaction period was 24 h in each batch reaction. Lipase was separated by filtration,

washed with petroleum ether, dried at room temperature, and then used in next batch reaction.

### 2.4 GC analysis

The wax, residue fatty acid and alcohol contents in the reaction mixture were quantified using a GC-2010 gas chromatography (Shimadzu Japan) equipped with a DB-1ht capillary column (30 m×0.25 mm; J&W Scientific, USA) and a flame ionizing detector (FID). The column temperature was held at 100 °C, heated to 180 °C at 15 °C·min<sup>-1</sup>, to 230 °C at 10 °C·min<sup>-1</sup>, finally to 330 °C at 20 °C·min<sup>-1</sup>, and then maintained for 5 min. The temperatures of the injector and detector were set at 350 and 360 °C, respectively. Heptadecanoic acid methyl ester purchased from Sigma was used as an internal standard.

### 2.5 Purification

Lipase was separated from reactant by filtration. The remaining mixture containing wax esters, cetyl alcohol and fatty acids in liquid layer was collected for further purification. Because the residual cetyl alcohol influences the low temperature property of wax esters and the fatty acid degrades the performance and stability of wax esters, the cetyl alcohol and fatty acids were removed. The fatty acids remained in the product was neutralized with saturated sodium carbonate solution, and the soap formed was removed by washing with water for two or three times [18]. Since the solubilities of wax esters and cetyl alcohol in ethanol are quite different, cetyl alcohol remained was removed by extracting with ethanol.

### 2.6 Determination of physical and chemical properties

Dynamic viscosity at 40 °C and 100 °C, flash point, solidifying point, pour point and acid value of the wax esters were tested according to industry standard methods [21, 23]. Properties of the wax esters obtained were compared with those of jojoba oil.

## 3 RESULTS AND DISCUSSION

### 3.1 Effect of lipase dosage

Lipase catalyzes esterification of long chain fatty acids and fatty alcohols to form corresponding wax esters under mild reaction conditions and may enhances the reaction rate at appropriate enzyme dosage. However, excessive lipase dosage may inhibit mass transfer and increase production cost [14]. Thus we optimize the lipase dosage in this part.

The effect of lipase dosage on esterification is shown in Fig. 1. Wax ester yield increases rapidly as the immobilized lipase amount increases from 4% to

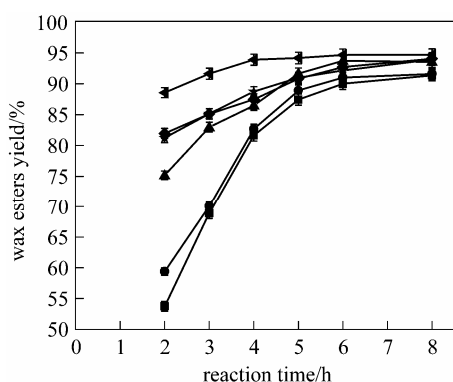


**Figure 1** Effect of lipase dosage on wax ester yield [Reaction conditions: oleic acid 2.00 g, acid/alcohol molar ratio = 1 : 0.9, temperature 40 °C, shaking at 170 r·min<sup>-1</sup>, reactor without stopper for water removal]  
lipase dosage/%: ■ 5; ● 6; ▲ 8; ◀ 10; ◆ 15; ★ 20

20% (by mass, immobilized lipase/oleic acid), but the increase is not obvious when the ratio is over 10%. Since excessive lipase dosage inhibits the mass transfer, the reaction rate is slower. Thus 10% (by mass, immobilized lipase/oleic acid) was selected in the subsequent experiments.

### 3.2 Effect of substrate molar ratio

Since the molar ratio of substrates is one of the most important factors that influence the product yield, the molar ratio was investigated. The acid/alcohol molar ratio was varied from 1 : 0.8 to 1 : 1.4. Fig. 2 shows that wax ester yield increases as acid/alcohol molar ratio decreases, but changes little when the ratio is lower than 1 : 0.9, indicating that cetyl alcohol inhibits lipase activity, which is consistent with that in previous study [15]. Table 1 shows the content of wax esters for reaction of 10 h. The wax ester yield is the highest (94.34%) when acid/alcohol molar ratio is 1 : 0.9, which was selected in the subsequent experiments.

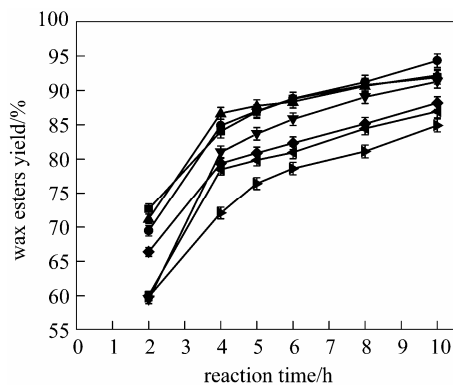


**Figure 2** Effect of fatty acid/cetyl alcohol molar ratio on the esterification [Reaction conditions: oleic acid 2.00 g, 10% (by mass) lipase dosage, temperature 40 °C, shaking at 170 r·min<sup>-1</sup>, reactor without stopper for water removal]  
acid/alcohol molar ratio: ■ 1 : 0.8; ● 1 : 0.9; ▲ 1 : 1; ▼ 1 : 1.1; ◆ 1 : 1.2; ◀ 1 : 1.3; ▶ 1 : 1.4

**Table 1** Contents of products after 10 h reaction analyzed by GC

| Fatty acid/cetyl alcohol molar ratio | Cetyl alcohol content/% | Fatty acid content/% | Wax ester content/% |
|--------------------------------------|-------------------------|----------------------|---------------------|
| 1 : 0.8                              | 0.38                    | 7.70                 | 91.92               |
| 1 : 0.9                              | 0.74                    | 2.41                 | 94.34               |
| 1 : 1                                | 3.87                    | 0.93                 | 92.20               |
| 1 : 1.1                              | 8.21                    | 0.49                 | 91.29               |

### 3.3 Effect of anhydration

Water content is a key factor that affects the activity of enzyme in a non-aqueous medium because lipases possess the unique activating feature at the interface between aqueous and organic phases [20]. However, too much water content would make lipase inactivation and inhibit wax ester synthesis [1, 24]. The reactions were performed in the flask with/without stopper, and the substrate molar ratio was 1 : 1 or 1 : 0.9. The excessive moisture produced during the reaction was removed by evaporation in the flask without stopper or absorbed by silica gel in the flask with stopper. Wax ester content at different reaction time is shown in Fig. 3. Wax ester yield is higher in open reactions than in closed reactions since the excess moisture can be removed in the open reaction, ensuring high wax ester yield (above 95%). Thus we selected the open reaction system in the larger scale reaction.

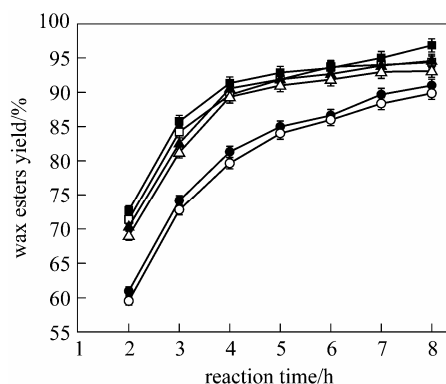


**Figure 3** Effect of open reaction on esterification [Reaction conditions: oleic acid 2.00 g, acid/alcohol molar ratio 1 : 0.9 or 1 : 1, 10% (by mass) lipase dosage, temperature 40 °C, shaking at 170 r·min<sup>-1</sup>]  
■ 1 : 0.9, open; □ 1 : 1, open; ▲ 1 : 0.9, closed, silica gel; △ 1 : 1, closed, silica gel; ● 1 : 0.9, closed; ○ 1 : 1, closed

### 3.4 Scale up and process of reaction

The volume of reactor was scaled up to 1 L three-neck flask, in which 10% (by mass) lipase dosage and 8 h reaction time were not suitable any more. Excessive lipase dosage (10% or 5%) resulted in mass transfer inhibition and lipase adhesion on reactor wall during the reaction. The reaction in the 1 L flask used agitation for mixing, so the shear force was larger than

that in the 50 ml reaction system without the agitation. The agitation caused stripping of the lipase from membrane, so the equilibrium time was longer than that in the 50 ml reaction system. Reaction rate is one of the key factors for the cost in industrialized production. Long reaction time will raise production cost and some side reactions may arise, but product quality can not be guaranteed if the reaction does not reach equilibrium.

The processes in the scaled up reaction with different lipase dosages and in the 50 ml flask with 10% (by mass) lipase dosage are shown in Fig. 4. The reaction reaches equilibrium at 8 h in the 50 ml flask and 24 h in the 1 L flask. The wax esters are hydrolyzed after 8 h in the 50 ml flask, so the optimum reaction time must be determined in industrialized production. 2.5% (by mass) lipase dosage is enough in the scaled up reaction and the wax ester yield reaches 95%, so the wax ester synthesis in this mode has industrialization prospect.

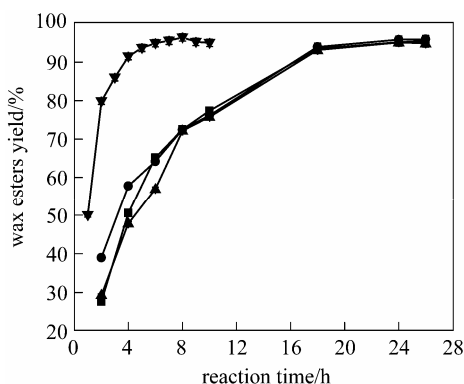


**Figure 4** Effect of lipase dosage and time on the reaction (Reaction conditions: oleic acid 300 g, acid/alcohol molar ratio 1 : 0.9, temperature 40 °C, shaking at 170 r·min<sup>-1</sup> in 50 ml flask, 200 r·min<sup>-1</sup> agitation speed in 1 L flask, reactor without cover for water removal) ■ 2.5%, 1 L flask; ● 5%, 1 L flask; ▲ 10%, 1 L flask; ▼ 10%, 50 ml flask

### 3.5 Repeated use of immobilized lipase

The cost of lipase accounts for a large part in the wax ester production. The main advantage of immobilization of enzyme is the repeated use over a long period of time [25]. To investigate the stability of the immobilized lipase, reaction was repeated several times. After completion of the reaction in 24 h, lipase was separated by filtration, washed with petroleum ether, dried at room temperature and used again in the same system. The result is shown in Fig. 5. The wax ester yield decreases significantly in the second cycle,

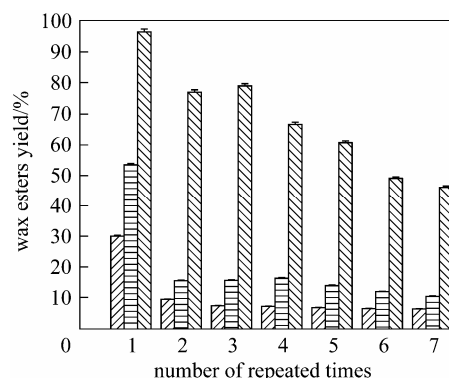


**Figure 5** Contents of product in each batch analyzed by GC

[Reaction conditions: acid/alcohol molar ratio 1/0.9, lipase dosage 2.5% (by mass), 40 °C, 200 r·min<sup>-1</sup> agitation speed, open reaction for water removal]  
 ▨ 2 h; □ 4 h; ▤ 24 h

from 96% in the first cycle to 81%. There is no obvious decrease in the yield in the next two cycles, which gives the yield of 76%–79%. However, the yield is only 46% in the seventh cycle, indicating that lipase activity decreases sharply. Thus the immobilized lipase could keep its activity for about 168 h in the agitation reaction system. Previous study showed that the lipase was used for 96 h (24 cycles) in a packed-bed reactor [17]. The lipase activity decreased more rapidly in stirred tank reactors may be due to the higher shear force.

### 3.6 Property test

The purified wax esters were analyzed by GC (Fig. 6), with purity of 96% cetyl oleate and residual cetyl alcohol content of less than 1%.

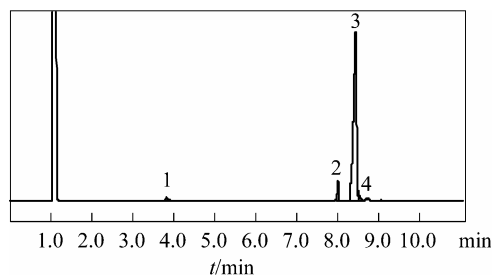


**Figure 6** GC analyses of wax esters after purification  
 1—cetyl alcohol; 2, 3, 4—wax esters

The properties of wax esters obtained were tested and compared with those of jojoba oil (Table 2). The

**Table 2** Properties of wax esters

| Performance indexes | Acid value/mg KOH·g <sup>-1</sup> | Dynamic viscosity/10 <sup>6</sup> /m <sup>2</sup> ·s <sup>-1</sup> |        | Pour point/°C | Solidifying point/°C | Flash point/°C |
|---------------------|-----------------------------------|--------------------------------------------------------------------|--------|---------------|----------------------|----------------|
|                     |                                   | 40 °C                                                              | 100 °C |               |                      |                |
| Jojoba oil          | <1.0                              | 17.1                                                               | —      | 23            | 20                   | 293            |
| the product         | 0.8                               | 18.6                                                               | 4.91   | 27            | 24                   | 279            |

parameters such as dynamic viscosity, flash point, solidifying point, pour point and acid value of wax esters are similar to those of jojoba oil. The high dynamic viscosity and flash point make wax esters suitable as compositions of lubricating oils, but the solidifying point and pour point need to be improved, e.g., replacing cetyl alcohol by oleic alcohol or alcohols with shorter chain lengths.

#### 4 CONCLUSIONS

Wax esters from oleic acid and cetyl alcohol could be synthesized in a solvent free system catalyzed by immobilized lipase from *Candida* sp. 99-125. The reaction conditions were optimized in a 50 ml flask incubated in a thermostatic cultivation cabinet at 40 °C and shaking at 170 r·min<sup>-1</sup>. The reactor was scaled up to 1 L three-neck flask with wax ester yield of about 95% under optimized conditions: acid/alcohol molar ratio 1 : 0.9, 2.5% (by mass) lipase dosage based on oleic acid, 24 h reaction time, and open reaction for water removal. The purity of the wax ester was 96% after removing free fatty acids and cetyl alcohol by saturated sodium carbonate solution and ethanol, respectively. The physical and chemical properties of wax esters are similar to those of jojoba oil, so the products could be used as a substitute of natural jojoba oil.

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