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Enzymatic synthesis of 2-ethylhexyl esters of fatty acids by immobilized lipase from *Candida* sp. 99–125

Xiang-ling He, Bi-qiang Chen, Tian-wei Tan*

Department of Biochemical Engineering, Institute of Chemical Engineering, Beijing University of Chemical Technology, Beijing 100029, PR China

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Abstract

The 2-ethylhexyl esters of fatty acids were synthesized by immobilized lipase from *Candida* sp. 99–125. The reuse stability of immobilized lipase was at least four batches. The conditions of enzymatic synthesis of 2-ethylhexyl palmitate were optimized. In the system of petroleum ether, 10% (w/w) immobilized lipase was used in the esterfication of 2-ethyl hexanol (7.8 mmol) and palmitic acid (7.8 mmol) at 40 °C with silica gel as the water absorbent. The esterification degree was 91% under these conditions. The purity of 2-ethylhexyl palmitate was 98% after purification consisting washing by water and evaporation to remove the organic solvent.

Keywords: Immobilized lipase; Enzymatic esterification; *Candida* sp. 99–125 lipase; 2-Ethylhexyl palmitate

1. Introduction

Fatty acid esters from 2-ethyl hexanol such as 2-ethylhexyl palmitate show attractions due to their applications in cosmetics, pharmaceutics, and food and chemical industries [1–3]. They are low temperature plasticizer for polyvinyl chloride, vinyl choride copolymers, polystyrene, ethyl cellulose and synthetic rubber, and also used in making lubricants and water-resistants or as solvents [4,5].

The methods for the production of 2-ethylhexyl palmitate based on chemical catalytic esterification have been described [6,7]. Chen et al. synthesized 2-ethylhexyl palmitate by chemical catalysis [6], which has a series of disadvantages, for example, many side-products and high energy consumption. Linko et al. studied enzymatic synthesis of mixture of 2-ethylhexyl esters of fatty acids by transesterification of rapeseed oil and 2-ethyl hexanol [8], in which monoesters, monoglycerides, diglycerides and triglycerides were included, and it is very difficult to separate 2-ethylhexyl palmitate from the mixture. Garcia et al. investigated the kinetics of palmitic acid esterification catalyzed by immobilized lipase Novozyme 435 to synthesize isopropyl palmitate, in which the reaction temperature was 65–75 °C, and the catalyst concentration was 2.2–7.83 wt.%. The esterification degree was about 70% in 120 min [9]. In this paper, synthesis of 2-ethylhexyl esters of fatty acids, especially 2-ethylhexyl palmitate, by immobilized lipase from *Candida* sp. 99–125 was studied. Finally, the optimal condition afforded a high esterification of 91% under 40 °C.

* Corresponding author. Tel.: +86-10-64416691; fax: +86-10-64715443.
E-mail address: tantw@buct.edu.cn (T.-w. Tan).

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2. Experimental procedure

2.1. Equipments

Gas chromatography GC4005A (Beijing East West Electrical Institute) with a packed column (2 m × 3 mm, 10% DEGS), GC data were recorded on intergrater (CR-5A, Shimadzu Co., Japan).

2.2. Materials

Butyric acid, hexanoic acid, caprylic acid, lauric acid, tetradecanoic acid, palmitic acid, stearic acid, eicosanic acid, decosoic acid, oleinic acid, 2-ethyl hexanol, petroleum ether (with analytical grade from Beijing Chemicals Factory). *Candida* sp. 99–125 was preserved in our laboratory.

2.3. The condition of the lipase fermentation

The fermentation was carried out in a 30 l reactor at 28 °C for 120–140 h, stirring with 300–500 rpm and 1 VVM air, and the culture medium was composed of 4 wt.% bean powder, 4 wt.% oil, 0.1 wt.% KH_2PO_4, and 0.1 wt.% $(NH_2)_2SO_4$. After the cells were removed by centrifugation, the lipase was precipitated by three volumes of acetone. The precipitation was washed by acetone and dried at the room temperature.

2.4. Analytical methods of 2-ethylhexyl palmitate

Reaction products were determined by gas chromatography (GC). The complete description of analytical method has been described in previous works [10].

2.5. Immobilization of lipase

The polyurethane foam was pre-soaked in two volumes of co-immobilization chemicals (w/v), containing: 5 g gelatin, 2 g lecithin, 2 g polyethylene glycol-6000 and 1 g magnesium chloride. Then the mixture was dried at room temperature. The treated dry support was used for immobilized lipase. The support (about 0.05–0.1 g) and the 1 ml solution of the lipase (about 400–800 U/ml) were mixed and dried overnight at room temperature.

2.6. Enzymatic synthesis of 2-ethylhexyl esters of fatty acids

The 2-ethylhexyl esters of fatty acids were synthesized in hexane system by immobilized lipase from *Candida* sp. 99–125 on the support by physical adsorption. A total of 2 mmol of oleic acid (0.565 g), 2 mmol of 2-ethyl hexanol (0.259 g) and 2 ml hexane were mixed, and then 0.03 g immobilized lipase (300 U) and 0.1 ml water were added into a 50 ml flask sequentially. The reaction was carried out in the flask at 40 °C for 24 h.

2.7. Enzymatic synthesis of 2-ethylhexyl palmitate

2-Ethylhexyl palmitate was synthesized in petroleum ether (60–90 °C) by immobilized lipase from *Candida* sp. 99–125. The 2-ethyl hexanol (7.8 mmol) and palmitic acid (7.8 mmol), and then 5 ml petroleum ether and 0.02 g immobilized lipase were added sequentially in 100 ml plugged conical flask. The mixture was shaken at 40 °C.

2.8. Measurement of esterification degree

The esterification degree was determined by titration with 0.1 mol/l NaOH to measure the palmitic acid or other fatty acids left in the reaction.

2.9. Purification of 2-ethylhexyl palmitate

The mixture, after reaction, was centrifuged at 4000 rpm (8 × *g* solution) for 10 min to recover the immobilized lipase. The palmitic acid left in the supernatant was neutralized with 0.5 mol/l NaOH, which formed aqueous and organic two phases. The organic phase was washed with one volume of water twice to remove 2-ethyl hexanol left. Then the petroleum ether in the organic phase was recovered by rotated evaporation. The final-product 2-ethylhexyl palmitate was obtained.

3. Results and discussion

3.1. Influence of different lipase

The esterification of 2-ethyl hexanol and palmitic acid was catalyzed by different lipases, including some

Table 1
Effect of different lipases on esterification

Lipase	Source	Amount of lipase (wt.%)	Esterification degree (%)
Immobilized procine pancreas	Sigma	5–10	<5
Lipolase 100T (immobilized)	Novo Co.	5–10	<1
Free lipase from *Candida* sp. 99–125	Fermented by our lab	2–4	<2
Immobilized lipase from *Candida* sp. 99–125	Fermented by our lab	5–10	>80

Remarks: 2–4% free lipase from *Candida* sp. 99–125 has the similar activity with 5–10% immobilized *Candida* sp. 99–125. Reaction conditions are shown in Section 2.7.

commercial lipases. Only the immobilized lipase from *Candida* sp. 99–125 could catalyze the reaction with the high conversion (Table 1), but the other lipases showed lower activity in this reaction. The reason maybe was the difference between specificity of the lipases. The first three lipases cannot catalyze the esterification of fatty acids and 2-ethyl hexanol. Furthermore, the immobilized lipase enlarged the interface of the reaction and improved the microenvironment for the enzymatic reaction.

3.2. Enzymatic synthesis of 2-ethylheyl esters of fatty acids

The reaction was carried out at 40 °C and pH 7.0. Among the 10 linear chain saturated fatty acids used, the fatty acids with carbon length of C_8–C_{16} had nearly the same esterification degree (about 85%), and the best one was obtained in case of caprylic acid. While the esterification degree of the fatty acids with carbon length of C_4, C_6, C_{20}, C_{22} was less than 50% (Fig. 1). The esterification of oleic acid was higher than the saturated fatty acids.

Among these esters of fatty acids, the 2-ethylhexyl palmitate has wide functions, so synthesis conditions of 2-ethylhexyl palmitate were studied in detail in this paper.

3.3. Enzymatic synthesis of 2-ethylhexyl palmitate

3.3.1. Influence of temperature and content of lipase

The influence of temperature on esterification is shown in Fig. 2. The optimal temperature was 40–50 °C (Fig. 2). Fig. 3 showed the influence of the

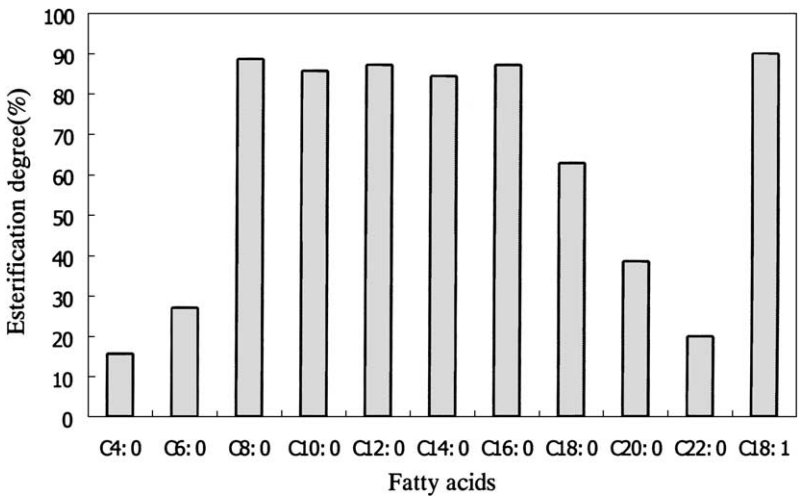

Fig. 1. Influence of fatty acids on esterification (reaction conditions are shown in Section 2.7; the content of the immobilized lipase was 10%).

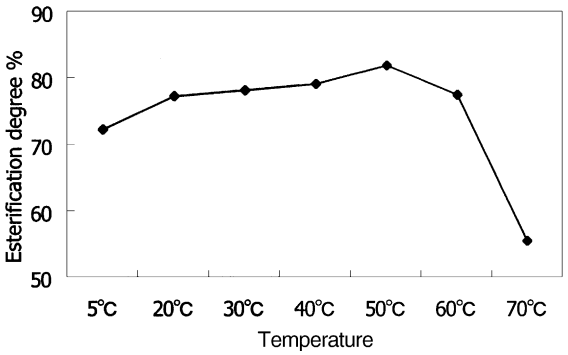

Fig. 2. Influence of the temperature on esterification (reaction conditions are shown in Section 2.7; the content of the immobilized lipase was 5%).

temperature and the content of the immobilized lipase on the reaction. At low immobilized lipase concentration (5 wt.% of acid), the conversion was found to increase with increasing temperature. However, if immobilized lipase was sufficiently (10 wt.% of acid) used, the conversion is similar at different temperature. The 10% immobilized lipase was excessive in this reaction so that at this time the temperature has not clear influence on the reaction. When 5% immobilized lipase was used to catalyze the esterification, the temperature had an obvious effect on the reaction. The lipase will lose the activity at the high temperatures, so 40 °C and 10% lipase is optimal point in the reaction.

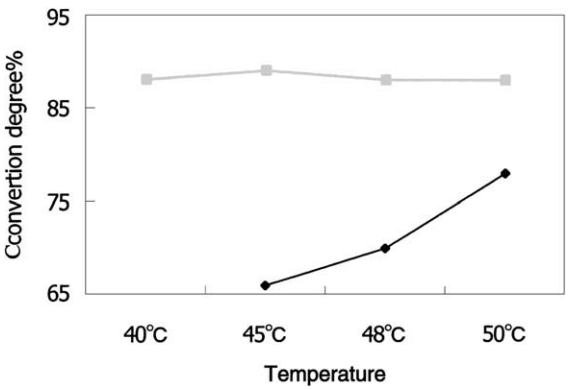

Fig. 3. Effect of temperature and enzyme concentration on esterification (◆) 5% immobilized lipase, (■) 10% immobilized lipase, the other reaction conditions are shown in Section 2.7.

3.3.2. Influence of solvent

Seven kinds of solvents were used in the esterification, including isooctane, petroleum ether (60–90 °C), *n*-hexane, *n*-heptane cyclohexane, *n*-pentane and no solvent. From results we can know that the types of solvents have considerable effects on esterification. Esterification degree in the system without solvent was the lowest (about 84%), and the highest esterification was obtained when isooctane was used as solvent (about 92%). When petroleum ether (60–90 °C) and *n*-hexane were used, the esterification was about 88%. Because palmitic acid is solid at room temperature, if solvent was added into the reaction system, the increase of solubility led to a conversion increased by 5–8%. On account of the cost and esterification degree, petroleum ether (60–90 °C) was the best solvent of the reaction.

The ratio of palmitic acid to petroleum ether (60–90 °C) also influenced the reaction. The esterification degree was the highest when the ratio was 1:5 (w/v) as shown in Fig. 4.

3.3.3. Influence of water adsorbent

Esterification is a reverse reaction of hydrolysis; the water produced by esterification inhibits further esterification. In the course of reaction, the water adsorbent was added into reactant. The effect on esterification is indicated in Table 2. When reaction proceeded at the 10th hour, the water adsorbent was added in, which led to an increase of esterification by 13%.

Among the water adsorbents including silical gel, molecular sieve, and Sephadex G-25, silical gel was the best because it was easily separated with lipase (Fig. 5).

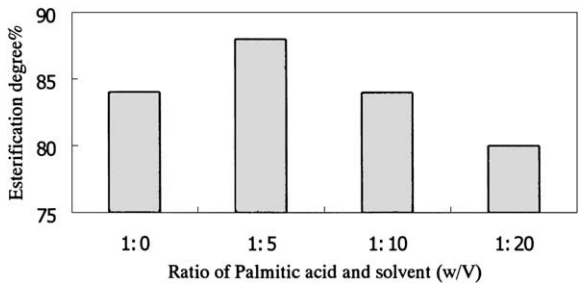

Fig. 4. Effect of the amount of solvent on the esterification (reaction conditions are shown in Section 2.7; the content of the immobilized lipase was 10%).

Table 2
Effect of water absorbent on esterification

Time adding into absorbent agent	Total time of reaction (h)	Esterification degree with water adsorbent (%)	Esterification degree of reaction without water absorbent (%)
10th hour	24	90	77
24th hour	48	91	85
0th hour	72	88	88
72nd hour	120	92	89

Remarks: in this table, water absorbent is silica gel (1 g). Reaction conditions are shown in Section 2.7.

3.3.4. *Influence of acid/alcohol molar ratio*

In the basic reaction system, acid/alcohol molar ratio was 1:1, but the effect of ratio on the result was shown in the Fig. 6. The best molar ratio of acid:alcohol was 2:1.

3.3.5. *The initial content of water in the reaction*

The content of water in the early stage of reaction had the direct effect on the esterification degree. The immobilized lipase was washed by cool acetone, then was centrifuged, and finally was dried in low temperature (−10 °C to −15 °C), in order to remove the water. Petroleum ether was also dehydrated by Na_2SO_4. The volume of the reaction system was 5 ml including 2-ethyl hexanol (0.5 g), palmitic acid (1.0 g), immobilized lipase (0.1 g), and petroleum ether. Before reaction, different contents of water were added in the system, and different esterification degrees were obtained (Fig. 7). When the content of water was 6 μl/ml (water/reaction system), the result was the best

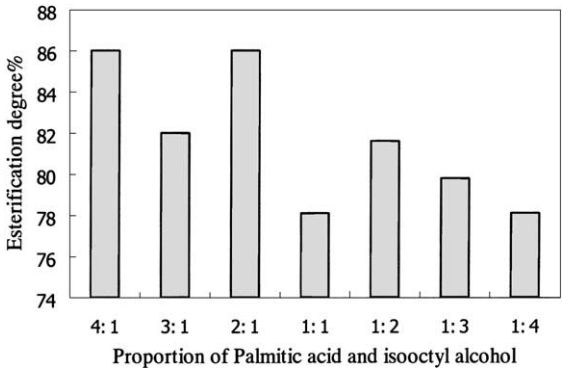

Fig. 6. Influence of substrate concentration on esterification (reaction conditions are shown in Section 2.7; the content of the immobilized lipase was 10%).

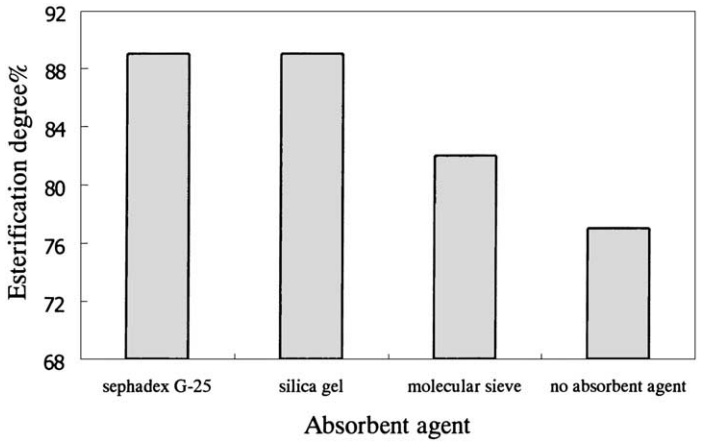

Fig. 5. Effect of type of absorbent agent on esterification basic reaction system, the absorbent was added at the 10th hour, and the reaction time 24 h.

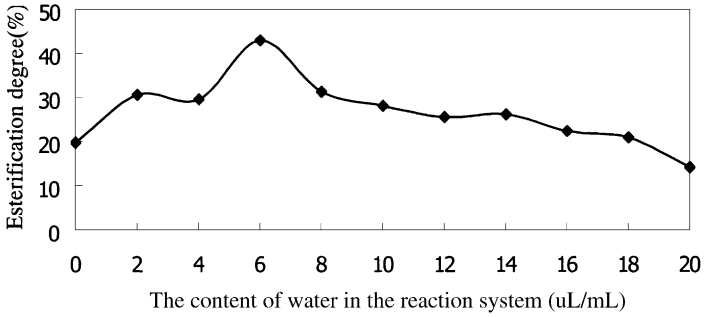

Fig. 7. Influence of the water content on the reaction esterification in the early stage (reaction conditions are shown in Section 2.7; the content of the immobilized lipase was 5%, the reaction time was 4 h).

(the reaction time was 4 h). This result indicated that suitable amount of water was necessary to maintain activity of lipases. But excess water did harm to the esterification.

3.3.6. Stability of immobilized lipase

The stability of immobilized lipase was improved when the support was treated by the method described in Section 2.6. The reaction of each batch was carried out at 40 °C for 24 h. From the first batch to the fourth batch, their esterification degrees were all around 90%. However, the esterification degree declined to 51% at the fifth batch. The reuse stability of immobilized lipase was at least four batches.

3.3.7. Preparation and purification of the product

The 2-ethylhexyl palmitate was purified with the method mentioned in Section 2.8. The palmitic acid left was neutralized with 1 mol/l NaOH, and the salt formed could be transformed into palmitic acid by acidification. The solvent was recovered.

The purity of 2-ethylhexyl palmitate was analyzed by GC. The end product was colorless and odorless liquid after purification, the purity of the 2-ethylhexyl palmitate was above 98%.

4. Conclusions

The 2-ethylhexyl esters of fatty acids were synthesized by immobilized lipase from *Candida* sp. 99–125. The stability of immobilized lipase was at least four batches. The 2-ethylhexyl palmitate was enzymatically synthesized from palmitic acid and 2-ethyl hexanol. The reaction was proceeded at 40 °C. The 10% (w/w) immobilized lipase was used and silica gel was added at the 10th hour. The petroleum ether was the optimal solvent of reaction, and the total reaction time was 24 h. The ratio of palmitic acid to the solvent was 1:5 (w/v). The esterification degree was 91% under the optimal conditions. The best molar ratio of acid to alcohol was 2:1. The reuse stability of immobilized lipase was at least four batches. The end product 2-ethylhexyl palmitate was obtained after purification with a purity of 98%.

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