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Full Length Research Paper

Synthesis of butyl acetate in *n*-heptane by the recombinant CS-2 lipase immobilized on kieselguhr

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Flavor esters are an important kind of flavor compounds, which are widely used in the food and pharmaceutical fields. The recombinant CS-2 lipase was immobilized after purification for which kieselguhr showed 18.5% of binding efficiency with 150.7 U/g of lipase activity. The immobilized lipase was used for the synthesis of butyl acetate in *n*-heptane. The factors affecting the synthesis of butyl acetate were investigated in the study. The results showed that the conversion of 98.2% was achieved under the following condition: reaction time (10 h); water activity (0.02); reaction temperature (55℃); the concentration of acetic acid and *n*-butanol (0.1 mol/L and 0.2 mol/L); the addition of 4 Å molecular sieve (0.5 g) at 8h of reaction time. The conversion of substrate decreased only from 98.2% to 87.4% after five cycles in use of the immobilized lipase.

Key words: The recombinant CS-2 lipase; Butyl acetate; *n*-Heptane; Immobilization

INTRODUCTION

Lipases (E.C.3.1.1.3) are among the most versatile classes of enzymes, which have been exploited for all kinds of reactions such as hydrolysis, esterification, transesterification, aminolysis, acidlysis and alcoholysis. In recent years, the examples of organic synthesis by lipase have dramatically increased, finding wide use for the production of pharmaceuticals, food additives or agrochemicals (Theil, 1995).

Lipase, when used in organic media, has shown some advantages: increased activity and stability; regiospecificity and steroselectivity; higher solubility of substrate and ease of products recovery; ability to shift the reaction equilibrium toward synthetic direction (Khmelnitsky and Rich, 1999). Therefore, the technological utility of lipase can be enhanced greatly by using them in organic solvents rather than its natural aqueous reaction media. Several research papers and reviews have been published to discuss application of lipases in organic media (Houde et al., 2004; Ghanem and Aboul-Enein, 2005; Hsieh et al., 2006).

Flavor esters are one of the most important components of natural flavors and fragrances. Currently, flavor esters are mainly produced by chemical synthesis and extracted from plant materials. However, polluting liquid acids as catalysts are used in the chemical method and post-treatment are required. Furthermore, flavor esters extracted from plant materials are expensive for commercial use (Salah et al., 2007). Indeed, flavor esters can be prepared by lipase and flavor esters obtained by enzymatic synthesis may be labeled as “natural” (Gillies et al., 1987). Thus, it is an alternative with high potential to traditional methods. Preparation of flavor esters by lipase from *Candida antarctica, Rhizomucor miehei*, *Rhizopus oryzae*, *Bacillus coagulans* have been reported (Larios et al., 2004; Salah et al., 2007; Krishna et al., 2000; Pahujani et al., 2008). Chen et al. 2011 investigated the synthesis of ferulic acid oleyl alcohol ester by lipase in an ionic liquid/isooctane system. Kumar and Kanwar 2011

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studied the synthesis of ethyl ferulate by a commercial lipase (Steapsin) immobilized onto celite-545 in a short period of 6h in DMSO. However, there is a clear lack of results regarding the synthesis of flavor esters by *Pseudomonas* sp. lipase in the specialized literature.

In the present study, the recombinant CS-2 lipase was used for immobilization onto kieselguhr. Moreover, the effect of various parameters on the synthesis of butyl acetate was investigated in order to obtain the optimum reaction conditions.

MATERIALS AND METHODS

Materials

HisTrap FF chromatography columns were from GE Healthcare. Kieselguhr (Celite-545) was purchased from Celite. All other chemicals were obtained from various commercial sources.

Organism

The recombinant *E. coli* BL21 (DE3) /pLysS harboring the genes of CS-2 lipase and its cognate foldase (the accession number in GenBank: GU220567) was used. The genes of CS-2 lipase and its cognate foldase were cloned from *Pseudomonas aeruginosa* CS-2.

Preparation of the recombinant lipase

The recombinant *E. coli* BL21 was precultured in a modified LB medium (adding 1% glucose) containing kanamycin (50 µg/ml) at 37°C with reciprocal shaking (190 r/min) overnight. The resulting cultures were transferred (1%, v/v) into TM medium (1.2% tryptone, 2.4% yeast extract, 1% glucose, 1% NaCl, 0.6%v/v glycerol) containing kanamycin (50 µg/ml) and grown at 37°C with shaking at 190 r/min until OD_{600} reached 1.0 (equivalent to 1.05×10^9 cfu /mL). The cells were induced with 0.05 mM isopropyl-1-thio-β-D-galactopyranoside and were grown at 20°C for 8 h after induction. The cells were harvested by centrifugation at 3,001 × *g* for 10 min and then were re-suspended in 50 mM Tris-HCl buffer (pH 7.9). The cells were disrupted by sonication. Cell debris was removed by centrifugation at 8,337× *g* for 10 min. The supernatant was loaded onto a HisTrap FF column equilibrated with 20 mM Tris-HCl buffer (pH 7.9) containing 500 mM NaCl and 10 mM imidazole. The column was then washed extensively with Tris-HCl buffer to remove unspecific and unbound proteins. The enzymes were eluted with 20 mM Tris-HCl buffer (pH7.9) containing 500 mM NaCl and 500 mM imidazole at a flow rate of 0.2 ml/min. The eluted fractions were collected and dialyzed to remove imidazole.

Immobilization of the recombinant lipase on kieselguhr

Purified lipase was reconstituted in Tris buffer. Kieselguhr (Celite-545) was washed three times with 0.15 M NaOH and 0.15 M HCl, respectively. Kieselguhr was then rinsed with deionized water and air-dried at room temperature. A fix amount of kieselguhr (5 g) was added to 50 ml lipase solution, and the mixture was stirred at 25°C under shaking condition (150 rpm). The immobilized lipase was filtered, rinsed three times with deionized water, and dried in a vacuum desiccator.

Effect of protein concentration and adsorption time on immobilization

Increasing amount of lipase was incubated with fixed amount of kieselguhr (5 g). The protein concentration was varied between100 µg/ml and 500 µg/ml. Activity of the bound lipase was assayed after immobilization. In order to determine the appropriate adsorption time, lipase was incubated with kieselguhr for different interval (from 0.5 h to 6 h). Activity of the bound lipase was checked after immobilization.

Protein assay

The protein concentration was determined by Bradford dye method using bovine serum albumin as standard protein (Bradford, 1976).

Assay of lipase activity

Lipase activity was assayed according to the method described previously (Peng et al., 2010). One unit of lipase activity was defined as the amount of enzyme liberating 1µmol *p*-nitrophenol per minute under standard assay conditions using *p*-nitrophenyl palmitate as a substrate.

Synthesis of butyl acetate by immobilized lipase in *n*-heptane

Acetic acid, *n*-butanol and *n*-heptane were dehydrated by 4 Å molecule sieves. The reaction mixture (10 ml) in a 50 ml sealed round bottom flask contained acetic acid, *n*-butanol and *n*-heptane. Synthesis of butyl acetate was performed under shaking condition (200 rpm) after the addition of 500 mg of immobilized lipase. Samples from the reaction mixture were withdrawn periodically and analyzed.

The effects of various parameters on ester synthesis were studied by varying water activity (0.02, 0.06, 0.11, 0.327, 0.528, 0.753,), temperature (40°C - 55°C), the concentration of acetic aid (0.05 mol/L - 0.4 mol/L), the concentration of *n*-butanol (0.05 mol/L - 0.4 mol/L) and adding 4Å molecular sieve during the reaction process.

Assay of acetic acid conversion

Acetic acid conversion was quantified by determining the residual fatty acid in the mixture, which was analyzed by volumetric titration (Leitgeb and Knez, 1990). All assays were performed in duplicates.

Acetic acid conversion (%) = (initial acetic acid concentrations

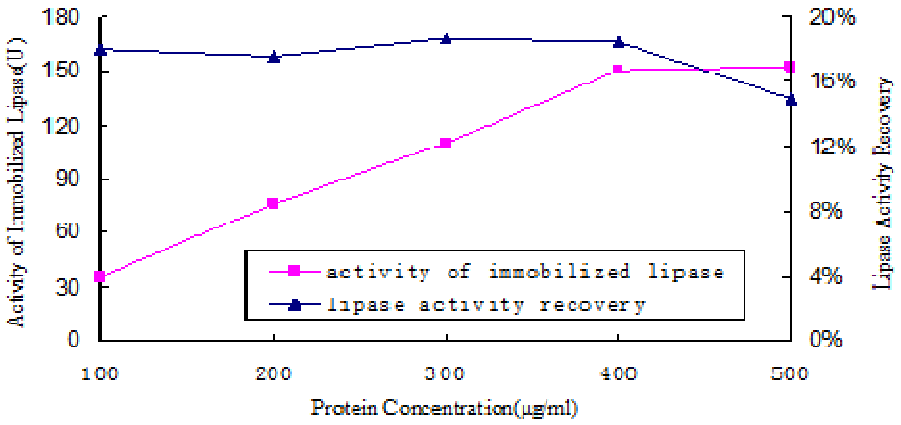

Figure 1. Effect of protein concentration on immobilization of the recombinant lipase onto Celite-545. The immobilization was performed at 25 ℃ with shaking at 150 rpm. The activity of immobilized lipase (squares) and lipase activity recovery (triangles) were determined with different amount of protein.

–residual acetic acid concentrations)/ initial acetic acid concentrations×100

Reuse of the immobilized lipase to synthesize butyl acetate in *n*-heptane

The immobilized lipase was used for five cycles for the formation of butyl acetate. After each use, the immobilized lipase was separated from the reaction mixture, washed three times with *n*-heptane and used for next cycle.

RESULTS AND DISCUSSION

Optimization of enzyme immobilization

Immobilization was a suitable approach to expose the enzyme more efficiently to the substrate, being especially indicated for synthetic processes in non-aqueous solvents (Persson et al., 2002). In present paper a method of adsorption was used to immobilize lipase. Figure 1 showed the activity of immobilized lipase increased with the given amount of protein. If the concentration of protein was higher than 400 μg/ml, the recovery of the lipase activity decreased because more and more lipase molecular remained in the solution. Therefore, kieselguhr showed the maximum lipase binding efficiency (18.5%) with 150.7 U/g of immobilized lipase activity. The adsorption kinetics showed that it took four hours to achieve optimum binding at 25 ℃ with shaking at 150 rpm (Figure 2). It was previously reported that the lipase from a *Bacillus* strain J33 was immobilized on silica gel G for 30 minutes, and 13% of total lipase added was retained on silica (Nawani et al., 1998).

Time course of butyl acetate synthesis

Time course study is a good indicator of enzyme performance and product yield. Figure 3 revealed that the conversion yield increased with reaction time. After 10 h, the yield reached a plateau, suggesting the equilibrium between synthesis and hydrolysis of ester was achieved. In a study by Pahujani et al., the synthesis of butyl acetate by lipase from *Bacillus coagulans* BTS-3 increased with an increase in the reaction time till 12h and started decreasing thereafter (Pahujani et al., 2007).

Effect of water activity (a_w) of the reaction system on ester synthesis

The amount of water in the system is one of the most important parameters for the synthesis reaction. The mixture of acetic acid, *n*-butanol and *n*-heptane was equilibrated at various water activity (a_w) in a closed chamber containing saturated salt solution, LiBr (a_w =0.06), LiCl (a_w =0.11), $MgCl_2$ (a_w =0.327), $Mg(NO_{3)2}$(a_w =0.528), NaCl (a_w =0.753), or 4 Å molecule sieves (a_w =0.02) for at least 24 hours.

The conversion percentage was found to decrease with increasing water activity (Figure 4). Maximum conversion was achieved at a water activity of 0.02. Many researches

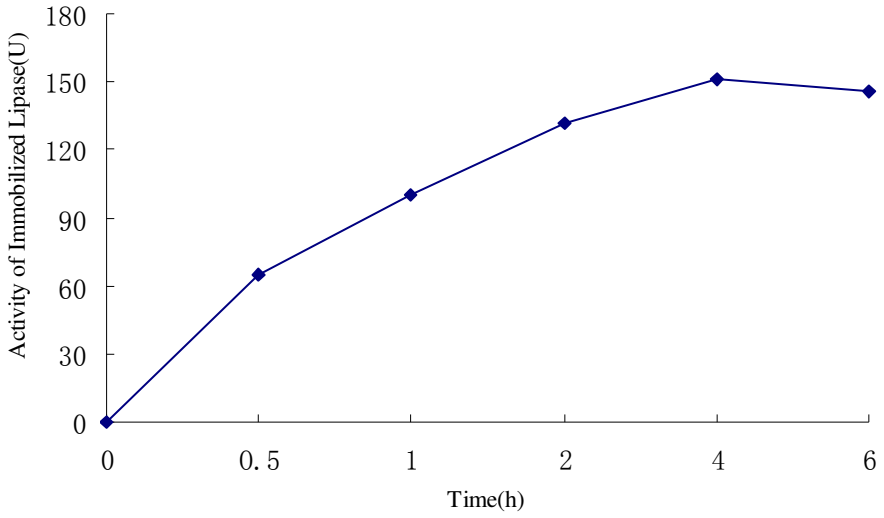

Figure 2 . Adsorption kinetics of the recombinant lipase onto Celite-545. The immobilization was performed at 25 ℃ with shaking at 150 rpm. The activity of immobilized lipase (squares) was determined with different immobilization period.

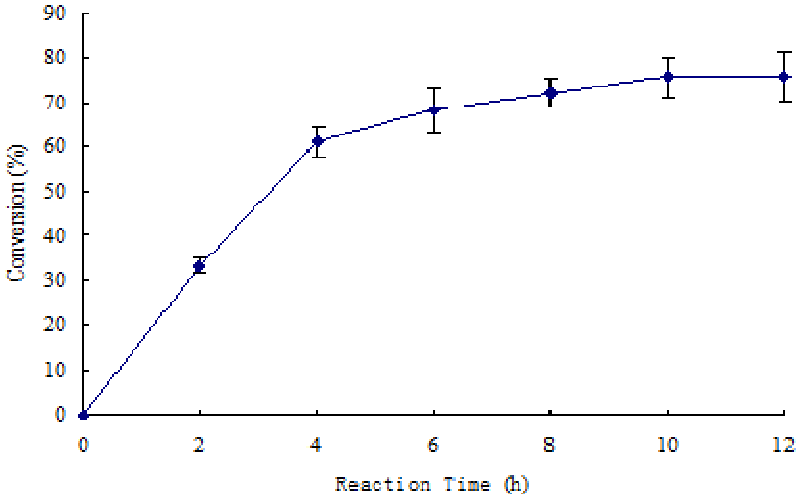

Figure 3. Time course of butyl acetate synthesis. The synthesis was carried out in *n*-heptane containing 0.2 mol/L acetic acid and 0.2 mol/L *n*-butanol in the presence of immobilized lipase (50 g/L) at 200 rpm and 50 ℃

indicated that lipase required essential water to sustain its conformation to prevent from the loss of activity. However, when a_w was too high, the binding of substrates and enzyme was hindered and the reaction was inhibited (Bell

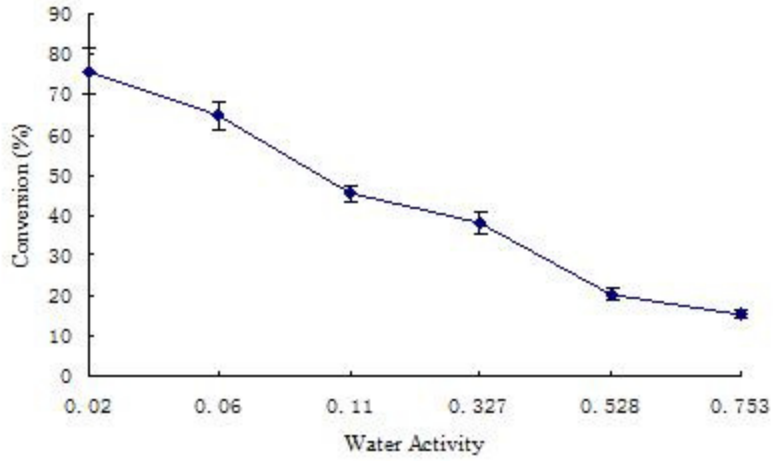

Figure 4. Effect of water activity (a_w) of the reaction system on ester synthesis. The synthesis was carried out in *n*-heptane containing 0.2 mol/L acetic acid and 0.2 mol/L *n*-butanol in the presence of immobilized lipase (50 g/L) at 200 rpm and 50 ℃ for 10 hours.

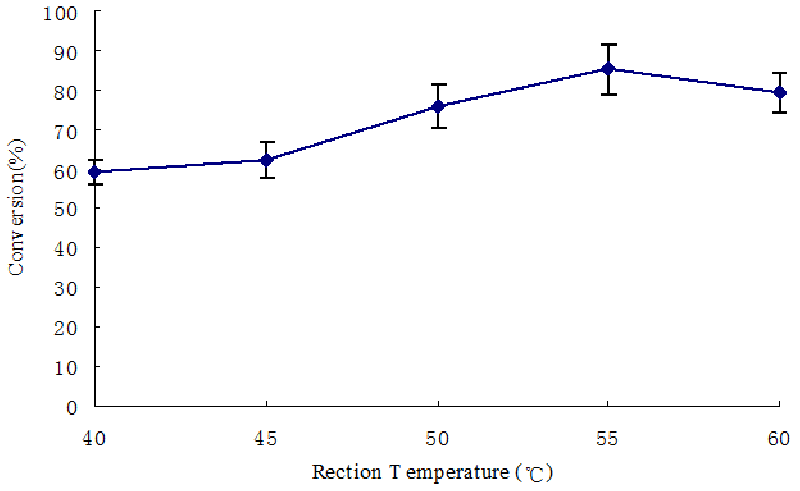

Figure 5. Effect of reaction temperature on ester synthesis. The synthesis was carried out in *n*-heptane containing 0.2 mol/L acetic acid and 0.2 mol/L *n*-butanol in the presence of immobilized lipase (50 g/L) at 200 rpm for 10 hours.

et al., 1995). Furthermore, water induced the hydrolysis of butyl acetate ester, decreasing the conversion yield.

Effect of temperature on ester synthesis

Reaction temperature affected the activity and stability of the lipase, and thus influenced the rate of reaction. Furthermore, temperature might have an effect on the porosity, hydrophobic character, and diffusion of reactants and/or products to the enzyme (Phillips, 1996). Figure 5 showed the temperature profile on the influence of temperature on the synthesis of butyl acetate within temperature range between 40 and 60℃. The percentage yield increased with increasing temperature from 40℃ to

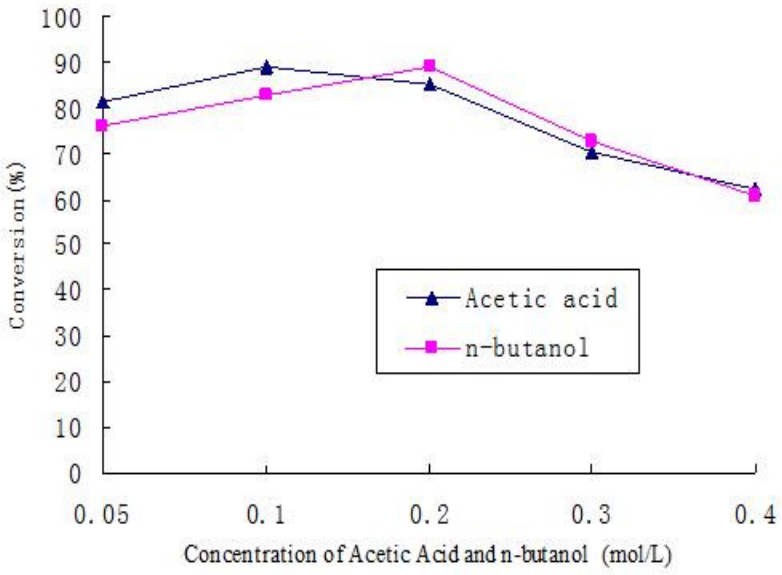

Figure 6. Effect of initial acetic acid concentration (triangles) and initial *n*-butanol concentration (squares) on ester synthesis. The synthesis was carried out in *n*-heptane in the presence of immobilized lipase (50 g/L) at 200 rpm and 55 ℃ for 10 hours.

55℃. A maximum conversion was achieved at 55℃. A further increase in the reaction temperature gave rise to the drop of product yield.

Effect of initial substrate concentration on ester synthesis

Figure 6 indicated that an increased concentration of acetic aid (0.05 mol/L- 0.1 mol/L) resulted in the improvement of product yield. However, when the initial concentration of acetic acid was higher than 0.1 mol/L, a decrease in the conversion yield was observed. The effect of initial concentration of *n*-butanol was also showed in Figure 6. The optimum concentration of *n*-butanol was 0.2 mol/L. Generally speaking, the esterification catalyzed by lipase fit into reverse reaction mechanism. In reaction process, the equilibrium shift to synthesis reaction with the increase of substrate concentration, which gives rise to the increasing yield. However, Substrate showed inhibitory effect on the enzyme if its concentration was too high (Mutua and Akoh, 1993).

Effect of addition of 4Å molecular sieve during the reaction process on ester synthesis

Ester synthesis enhanced by removing water produced form the reaction (Zaks and Klibanov, 1985). During the synthesis process, 4 Å molecular sieve (0.5 g) was added as water absorbent in the reaction system at the 8th hour. The conversion was increased to 98.2% after the addition of 4 Å molecular sieve. The result was due to the equilibrium shift to synthesis reaction with the removal of water produced in the reaction.

Reusability of immobilized lipase for ester synthesis

Repeated use of immobilized enzyme might help to drive down the product cost and make the enzymatic process economically viable. In this study, the immobilized enzymes were repeatedly used as the biocatalyst for synthesis reaction and subsequently recovered and reused. Figure 7 showed that percentage conversion only decreased from 98.2% to 87.4% after five cycles of reuse of the immobilized lipase. The results indicated the feasibility and suitability of the lipase from *Pseudomonas aeruginosa* CS-2 immobilized on Kieselgur for production of butyl acetate. In a study by Salah et al., the production of butyl acetate esters by immobilized *R. oryzae* lipase was achieved in the presence of heptane and hexane with conversion yields of 80% and 76%, respectively. On the other hand, Pahujani et al. reported that the lipase from *Bacillus coagulans* immobilized on nylon-6 achieved about 75% conversion of vinyl acetate and *n*-butanol into butyl acetate in *n*-heptane at 55℃ in 12 h.

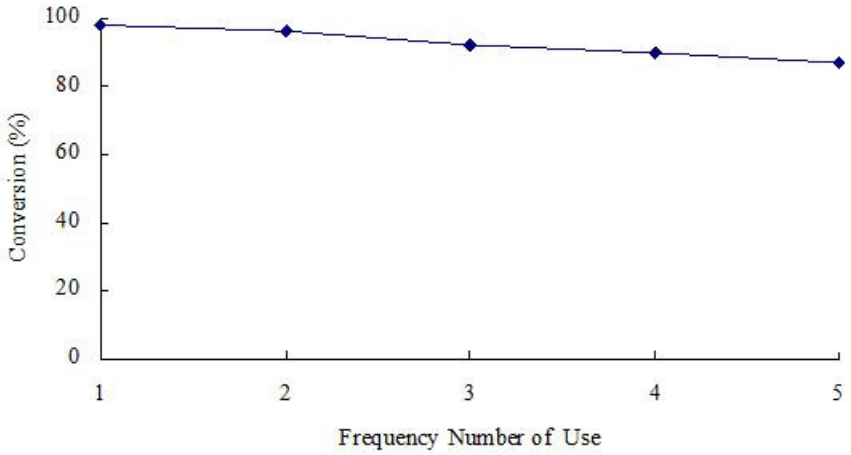

Figure 7. Reusability of immobilized lipase for ester synthesis. The synthesis was carried out in *n*-heptane containing 0.1 mol/L acetic acid and 0.2 mol/L *n*-butanol in the presence of immobilized lipase (50 g/L) at 200 rpm and 55 ℃ for 10 hours. During the synthesis process, 4 Å molecular sieve (0.5 g) was added as water absorbent in the reaction system at the 8th hour.

CONCLUSION

In the present study, a recombinant lipase was immobilized onto Celite-545 with 18.5% of binding efficiency and 150.7 U/g. The immobilized lipase efficiently synthesized butyl acetate in *n*-heptane containing 0.1 mol/L acetic acid and 0.2 mol/L *n*-butanol at 200 rpm and 55 ℃ for 10 hours, adding 4 Å molecular sieve as water absorbent in the reaction system. Moreover, the immobilized enzyme had good reusability for ester synthesis.

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