Engineering in Life Sciences

Eng. Life Sci. **2017**, *17*, 768–774 www.els-journal.com

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Research Article

Biocatalytic synthesis of vitamin A palmitate using immobilized lipase produced by recombinant *Pichia pastoris*

In this work, the *Candida antarctica* lipase B (CALB), produced by recombinant *Pichia pastoris*, was immobilized and used to synthesize vitamin A palmitate by transesterification of vitamin A acetate and palmitic acid in organic solvent. The reaction conditions including the type of solvent, temperature, rotation speed, particle size, and molar ratio between the two substrates were investigated. It turned out that the macroporous resin HPD826 serving as a carrier showed the highest activity (ca. 9200 U g^{-1}) among all the screened carriers. It was found that the transesterification kinetic of the immobilized CALB followed the ping pong Bi-Bi mechanism and the reaction product acetic acid inhibited the enzymatic reaction with an inhibition factor of 2.823 mmol L^{-1}. The conversion ability of the immobilized CALB was 54.3% after 15 cycles. In conclusion, the present work provides a green route for vitamin A palmitate production using immobilized CALB to catalyze the transesterification of vitamin A acetate and palmitic acid.

Keywords: *Candida antarctica* lipase / Immobilization / Kinetics / Transesterification / Vitamin A palmitate

Additional supporting information may be found in the online version of this article at the publisher's web-site

Received: July 21, 2016; *revised:* January 12, 2017; *accepted:* February 10, 2017

DOI: 10.1002/elsc.201600178

1 Introduction

Vitamin A is a group of unsaturated nutritional hydrophobic compounds, including retinol, retinal, and retinoic acid , that help visual cells to respond to weak light and the lack of vitamin A affects the dark adaptation ability of eyes. Vitamin A also functions as an antioxidant to maintain the normal form and the function of epithelial tissues, the development of normal skeleton, as well as the function of immune system [1]. Because vitamin A cannot be synthesized in the body, thus it must be obtained from external sources like food (i.e. dietary supplements or enriched foods). However, vitamin A can be easily decomposed particularly when being exposed to ultraviolet or in the presence of oxygen under natural conditions, thus vitamin A derivatives that possess better chemical and thermal stabilities have been developed as substitutes of vitamin A. Currently, two most common vitamin A derivatives—vitamin A acetate (VAA, about 90%) and vitamin A palmitate (VAP, about 10%)—have been frequently preferred in dairy products as an alternative source of vitamin A [2]. Among them, VAA is usually used as feed additive, while VAP has a better stability than VAA and is mainly used in high-end products, such as medicine or food additive [1, 3, 4].

Currently, commercial VAP is mainly obtained through chemical synthesis, a method that has been well developed [5].

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Abbreviations: CALB, *Candida antarctica* lipase B; **VAA**, vitamin A acetate; **VAP**, vitamin A palmitate

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However, the disadvantage of this method is that the process is relatively harsh because of the use of acid and alkali, which can easily destruct the starting raw materials. Furthermore, the intermediate vitamin alcohol is not stable, and the final product is very difficult to be separated and purified because the by-products always affect the stability of the end product. Another major flaw of the chemical synthesis is the energy consumption and pollution, which not only generate great economic costs, but also cause serious environmental pollutions. Therefore, seeking new types of green and environmentally friendly production mode is the current trend to obtain vitamin A derivatives. At this point, biosynthetic method is believed to be the first choice because it has the advantages of mild conditions, low energy consumption, low raw material requirements, and high-quality end-products. And the most attractive biosynthetic way of VAP production is lipase-catalyzed transesterification of VAA.

Lipase (EC 3.1.1.3) can catalyze the hydrolysis, synthesis, ester transferation, and transesterification of esters with a high regioselectivity and stereoselectivity. It has been widely applied in organic synthesis, chiral drug separation, food industry, and other fields [6]. *Candida antarctic* lipase B (CALB) is one of the most widely used lipase, and its structure and amino acid sequence have been determined by Uppenberg et al. [7]. CALB catalyzes both water soluble and nonwater soluble substances, thus has a wide range of potential applications. However, the natural CALB has a low enzymatic activity and requires a long fermentation period [8], therefore various genetic modifications have been conducted to improve the biotechnological applicability of CALB. CALB was first cloned and expressed in *Aspergillus oryzae* by Novozymes in 1995 [8] and latter expressed in the cytoplasm of *Escherichia coli* by Liu et al. [9]. A His tag was then introduced into the carboxyl-end of CALB by Blank et al. [10] to facilitate its purification. Finally, Jung et al. [11] improved the CALB expression in *E. coli* through codon optimization and introduction of hydrophobic amino acids into the surface of the enzyme. The thermal stability of CALB can be further enhanced through certain mutations and it can be displayed even on the surface of *Saccharomyces cerevisiae* [12] or *Pichia pastoris* [13]. However, because the substrate of lipase usually has low water solubility, the reaction rate is thus limited by the substrate concentration in aqueous solution. To overcome this restriction, organic solvents can be used to improve the substrate concentration as it was found that CALB shows catalytic activity in organic solvents under suitable conditions. Furthermore, it was found that CALB being immobilized on certain supporting materials still maintains its catalytic activity, which even shows a better stability and is amiable to be recovered [14]. Indeed, being immobilized on textile, recombinant lipase produced in *P. pastoris* [15] has been used for the biosynthesis of different vitamin A esters.

In our previous work, we have designed and synthesized the CALB gene according to the codon preference of *P. pastoris* and successfully expressed CALB in *P. pastoris* with an enzyme activity of 90 U mL^{-1} [16]. In the present work, we aimed to optimize the immobilization of CALB and investigate the effects of various parameters on the synthesis of VAP. Furthermore, the initial reaction rate, activation energy, various kinetic parameters, and the reusability of the immobilized enzyme were also determined.

2 Materials and methods

2.1 Enzymes and chemicals

The strain was constructed as described previously [16]. The porous resins were purchased from Qinshi technology Co., Ltd (Zhengzhou, China). VAA, palmitic acid, and VAP were kindly provided by Kingdomway Incorporated Company (China). PTM1 (trace elements) was purchased from QingDao Top Biotech Co., Ltd (QingDao, China). All other chemicals were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd (China).

2.2 Preparation of immobilized lipase

The fermentation process to produce lipase was as reported previously [16]. Briefly, PTM1 solution was prepared by adding 5 mL sulfuric acid and 95.0 g PTM1 to 1 L distilled water, and then boiled until the materials were completely dissolved. The culture medium was BSM ($C_3H_8O_3$: 40 g L^{-1}; 85% H_3PO_4: 26.7 mL L^{-1}; $CaSO_4 \cdot 2H_2O$: 0.93 g L^{-1}; K_2SO_4: 18.2 g L^{-1}; $MgSO_4 \cdot 7H_2O$: 14.9 g L^{-1}; KOH: 4.13 g L^{-1}; adjust pH to 5.0 by 28% NH_4OH; PTM1 solution: 4 mL L^{-1}). After sterilization, the temperature of BSM was adjusted to 30°C and the pH was adjusted to 6.0. After glycerol was exhausted, methanol (5% residual liquid volume) was added to induce lipase expression at 25°C. The enzyme activity reached >100 U mL^{-1} after 144 h.

The porous resin was added to the fermentation tank with the volume ratio of 1:20 for 18 h. The resins were separated by sifting through 100 meshes and then washed by deionized water for three times. The immobilized lipase was obtained after freeze-drying.

2.3 Lipase activity assay

The esterification reaction of lauric acid and 1-propanol was used to determine the lipase activity. The substrate solution was prepared by dissolving 24.0 g 1-propanol in 3.2 mL water and then heated to 60°C, and 80.1 g lauric acid (preheated to 60°C) was added gradually. The substrate solution was preserved in a 60°C water bath. Before reaction, 10 g substrate was put in a 50-mL conical flask; 50 mg lipase was added to initiate the esterification reaction. Then, the flask was sealed and the reaction was conducted at 60°C with magnetic stirring at 200 rpm; 20 min later, 10 μL sample was removed and combined with 1 mL 2% acetic acid/heptanes solution for GC analysis using an Agilent 7890A that is equipped with a flame ionization detector, an automatic sampler, and a J&W brand DB-WAX (30 m × 0.250 mm × 0.50 μm) column. The temperature of injection port (with a split ratio of 50:1) and flame ionization detector was both set at 300°C. The flow rate of carrier gas (nitrogen) was 1.0 mL min^{-1}. The oven temperature was kept at 60°C for 1 min

and then rised at a speed of 40°C per minute to 240°C and kept at 240°C for 15 min [17]. Injection volume of 1 μL was used for all samples. The peak areas of lauric acid and lauric acid ester were used for quantitative analysis by external standard method.

One unit (U) lipase activity was defined as the amount of immobilized CALB that is required to release 1 μmol of lauric acid ester per minute at operation conditions described above.

2.4 Synthesis of VAP

As shown in Fig. 1, VAP was synthesized by transesterification of VAA and palmitic acid. The reaction was performed in a 50-mL conical flask with a plug. Palmitic acid (0.234 g) and VAA (0.1 g) were first dissolved in 10 mL petroleum ether, and then 20 mg immobilized CALB was added to start the reaction at 35°C in a shaking incubator with an agitation speed of 200 rpm for 6 h.

2.5 Chromatographic analysis of VAP

Fifty microliters of reaction mixture was removed at regular intervals and diluted with methanol to 0.5 mL, of which 20 μL was analyzed by Agilent 1200 HPLC equipped with an Agilent G1328B DAD detector and an Agilent XDB-C18 chromatographic column. The mobile phase was 100% methanol. The oven temperature was kept at 30°C and the detection wavelength was 326 nm. The flow rate of mobile phase was 1 mL min^{-1} [18].

3 Results and discussion

3.1 Immobilization of lipase B

Five different types of resin were used as a carrier for the immobilization of lipase B. As shown in Table 1, HPD826 gave the highest activity. The lipase activity of the immobilized lipase followed the sequence as carrier HPD826 >HPD300 > HPD100> HPD600 >NKA-II. It was found that the nonpolar resins (HD300 and HPD100) give a higher activity than that of the polar resins (HPD600 and NKA-II). This indicates that the nonpolar interaction greatly contributes to the adsorption of the enzyme on the surface of the carrier. At the same time, no matter it is nonpopar or polar resin, the carrier with a higher specific surface area is preferred (HPD300 > HPD100, and HPD600 > NKA-II), which is likely due to the case that a higher specific surface area provides a higher enzyme loading ability. Anyway, the H-bond resin, HPD826, gives the highest activity, suggesting that the hydrogen bond interaction may play an important role in the adsorption of lipase on HPD826. The activity of immobilized lipase adsorbed on the carrier HPD826 was about 9200 U g^{-1}, which has a very close activity in comparison with commercial lipase–Novozym 435 (ca. 10 000 U g^{-1}). However, we believe that the preparation cost of the immobilized lipase (< 100 \$ kg^{-1}) is much lower than that of Novozym 435 (>4000 \$ kg^{-1}). Therefore, it is obvious that the preparation of the immobilized enzyme as described in this work is much more cost effective.

3.2 Influence of reaction media

Organic solvent that is able to solubilize the substrate well but does not affect the catalytic ability of the enzyme is usually used in the enzymatic reaction. Laane et al. [19] used an organic solvent polarity parameter logP, which refers to the logarithms of the distribution coefficient of the organic solvent in the octanol/water system, to describe the influence of organic solvents on the enzyme reaction. In the present study, our results showed that hydrophobic solvents such as petroleum ether have a good compatibility for CALB because it resulted in a conversion of 81% after 6 h (Supporting Information Table 1). This may be attributed by a better maintain ability of microaqueous system in hydrophobic solvents, thus guarantees the conformational mobility of enzyme molecules [20]. In contrast, the microwater system around the enzyme can be easily changed in hydrophilic solvents, thus resulting in a decreased conversion [21]. The higher of the hydrophobicity of the solvents, the weaker of their ability to attract water molecules that are surrounding the enzyme, therefor the more water can be retained on the surface of the enzyme molecules, that is, a greater enzymatic activity [20–22]. Thus, petroleum ether was selected as the solvent for further studies.

3.3 The influence of temperature

It has been reported that the immobilization can improve the thermal stability of enzymes; however, the immobilized enzyme are more easily susceptible to thermal denaturation [20, 22]. In addition, considering temperature is a key parameter that affects the solubility of the reactants within the system and promotes the collisions between molecules [22, 23], the optimal temperature that is required for the immobilized CALB to catalyze the reaction should be determined. As shown in Fig. 2, the initial reaction rate gradually increases with the increase in temperature. But the conversion reaches a maximum value at 35°C, and a further increase in temperature from 35 to 50°C leads to a decrease of conversion, which is probably caused by the increase in inverse reaction rate.

The apparent activation energy (E_a) was calculated on the basis of ln(initial rates) versus reciprocal of temperature in the range of 25–50°C [22, 23]. The activation energy obtained from the Arrhenius curve (data are shown in Supporting Information Fig. S1) was 5.96 kcal mol^{-1}. This value is close to the activation energy (5.2 kcal mol^{-1}) of the esterification reaction of 3-(2-methylphenoxy) propane-1,2-diol [24].

3.4 Influence of rotation speed

Considering rotation speed can influence the diffusion of substrate from bulk liquid phase to immobilized enzyme, that is, external diffusion, thus the influence of rotation speed on enzymatic reaction was examined. It was found that the relative

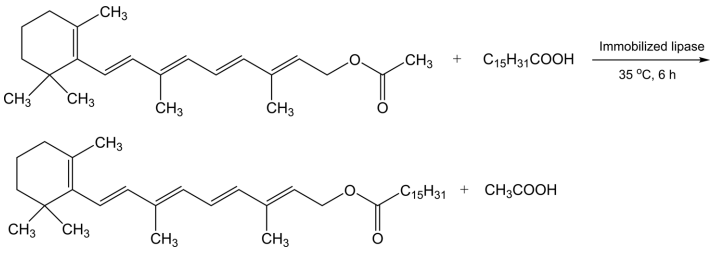

Figure 1. Vitamin A palmitate synthesis catalyzed by immobilized lipase CALB.

Table 1. The activity of immobilized lipase B on various carriers with different physical properties

Carrier	Type of skeletons	Particle size (mm)	Average pore size (Å)	Specific surface area ($m^2\ g^{-1}$)	Polarity	Activity ($U\ g^{-1}$)
HPD826	Styrene	0.3–1.2 (>90%)	90–100	500–600	H-bond	9234 ± 275
HPD300	Styrene	0.3–1.2 (>90%)	50–55	800–870	Nonpolar	6413 ± 158
HPD100	Styrene	0.3–1.2 (>90%)	85–90	650–700	Nonpolar	5925 ± 157
HPD600	Styrene	0.3–1.2 (>90%)	80	550–600	Polar	3963 ± 126
NKA-II	Styrene	0.3–1.2 (>90%)	145–155	160–200	Polar	2208 ± 104

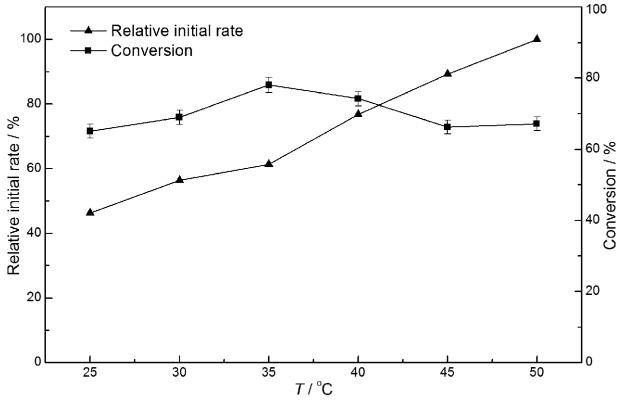

Figure 2. The influence of temperature on the initial reaction rate and conversion of vitamin A acetate transesterification. Reaction conditions: vitamin A acetate, 0.100 g; palmitic acid, 0.234 g; solvent (petroleum ether), 10 mL; immobilized lipase 0.02 g; rotation speed, 200 rpm; time, 6 h for conversion, 10 min for initial rate.

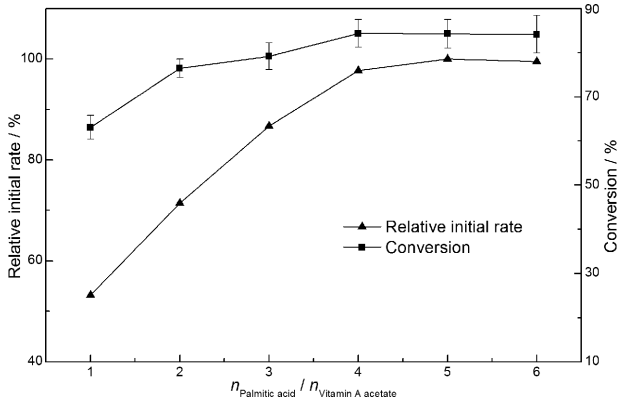

Figure 3. The effects of molar ratio on initial reaction rate and conversion for vitamin A palmitate transesterification. Reaction conditions: temperature, 35°C; rotation speed, 160 rpm; other conditions are the same as in Fig. 2.

reaction rate increased with rotation speed from 100 to 160 rpm, but the reaction rate did not change significantly when the rotation speed increased from 160 to 210 rpm (data are shown in Supporting Information Fig. S2). This suggests that the influence of rotation speed above 160 rpm on reaction rate can be ignored.

3.5 Influence of particle size

Considering the internal diffusion of substrate may have a great influence on the reaction rate for porous catalyst [25, 26], so the reaction rate of immobilized CALB with different sized particles was tested. It was found that the reaction rate increased when the diameter of immobilized CALB decreased from 0.9 to 0.4 mm (data are shown in Supporting Information Table 2), while a further decrease in the particle size from 0.4 to 0.2 mm did not have a significant influence on the reaction rate. In conclusion, the influence of internal diffusion is eliminated with the particle size smaller than 0.4 mm and the immobilized CALB with particle size 0.3–0.4 mm was used in the following kinetic experiments.

3.6 The effects of molar ratio on reaction

The transesterification reaction between VAA and palmitic acid is reversible, therefore it is expected that increasing the concentration of cheaper substrate palmitic acid can improve the conversion of VAA from the perspective of chemical equilibrium [27, 28]. Thus, the reaction was conducted using different molar ratio of palmitic acid and VAA. In 10 mL petroleum ether, the amount of VAA was fixed at 0.1 g, and the amount of palmitic acid was changed from 0.078 to 0.390 g (Fig. 3). It can be observed that the conversion rate increased with the increase in

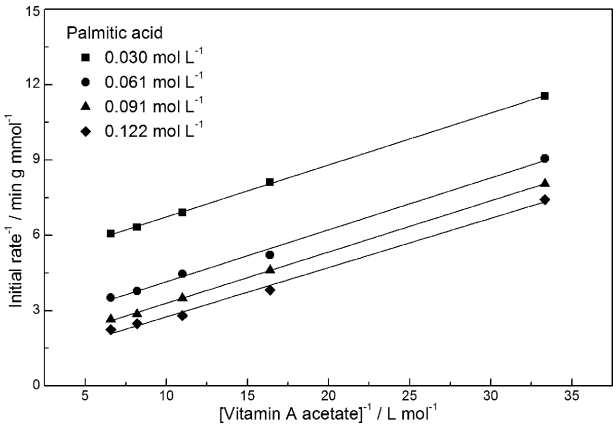

Figure 4. Lineweaver–Burk plot. Reaction conditions: temperature, 35°C; rotation speed, 160 rpm; other conditions are the same as in Fig. 2.

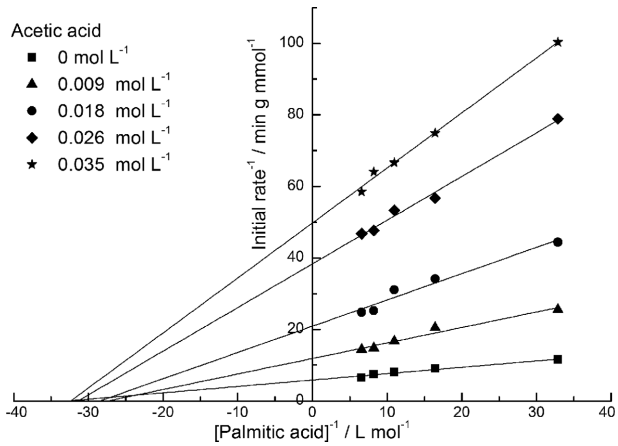

Figure 5. The inhibition kinetics of acetic acid on the lipase CALB activity. Reaction conditions: temperature, 35°C; rotation speed, 160 rpm; other conditions are the same as in Fig. 2.

the substrate molar ratio. When the ratio of palmitic acid and VAA was set at 4:1, the conversion rate reached 84%. But a further increase in the molar ratio does not further improve the conversion rate.

3.7 Kinetics and reaction mechanism

Considering the importance of the reaction kinetics for the reactor design and scaling up as well as the case that lipase catalyzed esterification usually follows the order Bi-Bi or ping pong Bi-Bi mechanism [28, 29], we determined the initial reaction rates using different VAA concentrations combined with different fixed concentrations of palmitic acid. The reciprocal of initial reaction rate versus the concentration of VAA (Lineweaver–Burk plot) was plotted and shown in Fig. 4; the initial reaction rate increases with the increase in substrate concentration in the range of 0.03–0.152 mol L^{-1} without any observed substrate inhibition. At different concentrations of the palmitic acid, the double reciprocal curves of the concentration of VAA and the initial reaction rate is in parallel, suggesting that the reaction in consistent with the ping pong Bi-Bi mechanism [30]. The kinetic parameters were obtained by the use of polymath 6.0 nonlinear regression analysis. The kinetic equation is as follows:

$$v = \frac{6.913 C_A C_B}{C_A C_B + 0.951 C_A + 1.101 C_B} \quad (1)$$

where v is the initial reaction rate, and C_A and C_B are the concentration of VAA and palmitic acid.

The obtained kinetic parameters were close to that of the esterification reaction of 3-(2-methylphenoxy) propane-1,2-diol that is catalyzed by the same lipase [24]. In addition, based on statistical parameters, a correlation coefficient of $R^2 = 0.983$ was obtained, indicating that the ping pong Bi-Bi mechanism fits well with the experimental data.

When the concentration of VAA was fixed, the equation was simplified to simple Michaelis–Menten equation of single substrate as follows:

$$v = \frac{v_m C_B}{K_m + C_B} \quad (2)$$

where v_m is the maximum reaction velocity and K_m is the Michaelis constant.

While setting the concentration of VAA at 0.03 mol L^{-1}, the Lineweaver–Burk plot of the initial reaction rate and the concentration of palmitic acid at various concentrations of acetic acid are shown in Fig. 5. It can be found that the product acetic acid has an inhibitory effect on the reaction. The initial reaction rate decreased with the concentration of acetic acid ranged between 0 and 0.035 mol L^{-1}. At different fixed concentrations of acetic acid, the double reciprocal curves of the concentration of palmitic acid and the initial reaction rate approximately intersect at the same point of horizontal axis, suggesting that the product acetic acid has a noncompetitive inhibition effect on the reaction. The kinetic equation can be expressed by the following formula:

$$v = \frac{v_m C_B}{(1 + C_I/K_I)(K_m + C_B)} = \frac{v_{m,app} C_B}{K_m + C_B} \quad (3)$$

where C_I is the concentration of acetic acid, K_I is the inhibition constant, and $v_{m,app}$ is the apparent maximum reaction velocity.

By plotting the slopes ($= K_m/v_{m,app}$) against the concentration of acetic acid, the product inhibition constant K_I was determined to be 2.823 mmol L^{-1} (data are shown in Supporting Information Fig. S3). Taking the inhibition of acetic acid into account, thus the kinetic equation can be expressed as follows:

$$v = \frac{6.913 C_A C_B}{\left[1 + C_I/\left(2.823 \times 10^{-3}\right)\right] \cdot (C_A C_B + 0.951 C_A + 1.101 C_B)} \quad (4)$$

3.8 The reusability of lipase

A main benefit of enzyme immobilization is recyclability, which is a vital factor to achieve an economical and practically feasible

catalytic process [31–33]. Therefore, the recovery of the immobilized CALB that was reused cycle by cycle was studied under the optimized reaction conditions (VAA, 0.100 g; palmitic acid, 0.312 g; solvent, 10 mL; immobilized lipase, 0.020 g; temperature, 35°C; rotation speed, 160 rpm; time, 6 h). After one cycle, the lipase was rinsed with petroleum ether for three times to remove potential trace products or reactants then stored at 4°C until being reused for the next cycle. It was found that the immobilized lipase retained the conversion ability of 54.3% after 15 cycles (Supporting Information Fig. S4). The reduction of conversion ability may be due to the conformational changes of the enzyme derived from continuous exposure to petroleum ether and substrates in each cycle [21, 29].

4 Concluding remarks

In the present work, lipase CALB was successfully immobilized on the ecofriendly resin HPD826. The reaction parameters such as reaction medium, effect of temperature, rotation speed, particle size, molar ratio of substrates, and reusability were systematically studied. Meanwhile, the immobilized lipase activation energy (E_a) was determined to be 5.96 kcal mol^{-1}. Nonlinear regression analysis of various kinetic parameters showed that the reaction is consistent with the mechanism of ping pong Bi-Bi. It was also confirmed that acetic acid has an inhibitory effect on the reaction. In particular, the immobilized enzyme shows an excellent recyclability. In conclusion, our data not only provide an important piece of information on the production and industrial application of CALB, but also represent a valuable reference to obtain a green synthesis process of VAP.

This work was funded by grants from the Science and Technology Program of Xiamen, China (No. 3502Z20143008) and the State Key Program of National Natural Science Foundation of China (No. 21336009).

The authors have declared no conflict of interest.

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