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Carica papaya lipase-catalyzed synthesis of terpene esters

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ABSTRACT

An efficient approach to synthesize terpene esters using plant lipase-*Carica papaya* lipase (CPL) as the biocatalyst was developed in this work. The effects of chain length of acyl donor and solvent type on the CPL-catalyzed transesterification reaction were investigated firstly. It was found that CPL showed the highest activity in *n*-hexane with vinyl octanoate as the best acyl donor. To obtain high yield of terpene esters, the main reaction parameters were studied and further optimized by response surface methodology. Ping–Pong Bi–Bi mechanism with dead end complex of citronellol was found to fit the initial rate data and the kinetic parameters were obtained by regression analysis. The optimal conditions were: 55 °C, 9% (w/w) of CPL based on substrate, equimolar ratio of substrates. Under these conditions, yield of more than 99% was achieved after 8 h reaction. Ionic liquids (ILs) were used to improve the operational stability because the CPL was found to lose its activity markedly during the repeated runs, it showed that the stability of CPL increased about 5 times when it was coated with ionic liquids. The CPL is low cost yet effective, thus the process developed here shows obvious potential for the production of terpene esters industrially.

1. Introduction

Esters of terpene alcohols, such as geraniol, linalool and citronellol are among the most important flavor and fragrance compounds used in the food, beverage, cosmetic and pharmaceutical industries (e.g. citronellyl acetate and citronellyl propionate are used in perfumery for fresh fruity rose odor). These flavor compounds are traditionally provided by extraction from natural sources or chemical synthesis [1]. However, natural flavor esters extracted from plant materials are either too scarce or expensive for commercial use, and the production of terpene esters via chemical synthesis normally involves catalysis by strong acids, this method has certain drawbacks such as formation of undesirable by-products, which can have an adverse effect on the characteristic odor of terpene esters and the esters are not considered as natural products. Thus, their market value is less than esters from natural sources.

The disadvantages, i.e., expense of isolation, shortage of supply and high cost of natural materials, negative impact associated with the word "synthetic or "artificial", etc., have led industries to seek new methods for producing natural flavor compounds. One approach is to produce these flavor esters using terpene alcohols by biotechnology [2]. Both geraniol and citronellol are available from essential oils and low cost, their esters which can be gradually released over long periods of time or in a controlled manner, should be easily obtained using acetic, propionic, hexanoic acid or octanoic acids with lipases as biocatalysts [3]. For example, Akoh and Yee [4] used *Candida antarctica* SP435 as biocatalyst to synthesize terpene alcohol esters and yield of 97.7% for geranyl acetate was observed after 8 h reaction.

Enzymatic synthesis of flavors is low temperature reactions requiring little energy and it is considered "natural" [5]. In the past decade, different lipases (such as *C. antarctica* lipase B and *Candida rugosa* lipase) had been successfully used as biocatalysts for the production of terpene esters by direct esterification, alcoholysis and transesterification reaction [4a,4b,6–9]. However, there is one bottleneck in enzymatic approach for terpene esters production. That is the high cost of lipase and its short operational life caused by the negative effects of solvent, temperature and so on. In order to develop a more effective enzymatic approach for terpenyl esters production, *Carica papaya* lipase, a very cheap plant lipase, was investigated as the biocatalyst for terpenyl ester synthesis in this work.

C. papaya is a soft-stemmed and unbranched tree able to grow up to 20 m in height. As a native to the Central America, the papaya tree has successfully established in many ecological niches in tropical and subtropical climates [10]. All aerial parts of the plant, including the unripe fruits present laticifers. Consequently, if incisions

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are made in those aerial parts, especially in the unripe fruits, an abrupt release of latex (the so-called *C. papaya* latex) is observed. The latex contains about 15% of dry matter, 85% water and varieties of hydrolytic enzymes. The soluble latex of *C. papaya* is a rich source of the cysteine endopeptidases, including papain, glycyl endopeptidase, chymopapain and caricain, which constitute more than 80% of the whole enzyme fraction. Papain (EC 3.4.22.2) is a minor constituent (5–8%) among the papaya endopeptidases as stated in our previous work [11]. The enzyme is used widely as meat tenderizer, and has also several other applications, e.g. for defibrinating wounds, treating edemas and shrink proofing of wool. The insoluble dry matter in the latex performs the activity of lipase and it is traditionally considered as "naturally immobilized" *C. papaya* lipase (CPL). In the past few years, CPL had been successfully used in lipids modification [12], asymmetric resolution of chiral acids [13], enantioselective production of nonnatural α-amino acids and resolution of secondary alcohols [14,15]. The annual production of papain reached up to hundreds of tons in China. Therefore, as a waste of papain production, CPL could be economically large-scale produced by simple purification process. In contrast, microbial lipases were obtained by complex steps including fermentation, purification and immobilization. As a result, they were costly for industrial use. Accordingly, the efficient "naturally immobilized" CPL held great potential to be cost-effective lipase for industry.

In this work, we tried to develop an efficient and economic approach to synthesize terpene esters using CPL as the biocatalyst. Firstly, different solvents, terpene alcohols and acyl donors were compared by determining their effects on yield of terpene ester, and the optimal solvent, terpene alcohol and acyl donor were used for further study followed by study of the reaction parameters. Then, the response surface methodology (RSM) and the 5-level-4-factor central composite design (CCD) were used to identify the factors that influenced the transesterification reaction of terpene alcohol to terpene esters and to verify whether any changes should be made in their settings to improve the reaction. The last part of the study dealt with the improvement of the operational stability of the CPL. Since the organic solvent and acetaldehyde produced by vinyl ester had been reported to inactivate the lipase [16–18], room temperature ionic liquids were tested for terpene ester production with respect to their effects on the CPL activity and stability.

2. Materials and methods

2.1. Materials

Crude *C. papaya* lipase was obtained from Shanghai Bairui Biotech Co., Ltd. Before use it was purified by the procedures: to 20 g of the crude *C. papaya* lipase was added 200 mL deionized water at 4 °C with gentle stirring for 30 min. The resultant solution was centrifuged at 10,000 rpm for 15 min and the supernatant was discarded. This procedure was repeated three times. The remaining precipitate was then collected and fast frozen using liquid nitrogen, then lyophilized for 8 h. This preparation was used as CPL in this work.

The ionic liquid coated CPL was prepared as follows: 100 μL of [bmim] [PF_6] (obtained from Lanzhou Institute of Chemical Physics, China) was added into a 3 mL test tube, followed by addition of 1.5 mL acetonitrile. Then 100 mg of CPL was added and the mixture was gently stirred for 10 min at room temperature. Finally, the acetonitrile was eliminated by continuous bubbling of N_2 for 30 min at room temperature. The resulting ionic liquid coated CPL particles were equilibrated to 0.32 a_w by over saturated $MgCl_2$ solutions in a closed container at 4 °C for 48 h, and then used as biocatalyst for terpene esters synthesis.

Geraniol, citronellol and linalool were kindly donated by Firmenich Inc (China). Vinyl acetate, vinyl butyrate and vinyl octanoate were bought from Tokyo Kasei Kogyo Co., Ltd. All other chemicals and reagents were obtained commercially and were of analytical grade.

2.2. General transesterification reaction

In a typical experiment, 3 mmol terpene alcohol and 3 mmol acyl donor (the molar ratio alcohol/acyl donor was 1:1) were dissolved in 4.0 mL of solvent in a 25 mL screw capped vial, followed by addition of 50 mg CPL. The reaction mixture was stirred at 200 rpm and 45 °C. Progress of the reaction was monitored by withdrawing aliquots of the reaction mixture for gas chromatography (GC) analysis.

2.3. GC analysis

GC analysis was performed on an Agilent 6890 Gas Chromatograph equipped with a flame ionisation detector (FID) and a HP-5 capillary column (5% phenyl methyl siloxane capillary, 30.0 m × 320 μm × 0.25 μm nomimal). The column temperature was kept at 100 °C for 0.5 min, heated to 160 °C at 10 °C/min, and then maintained for 1 min. The temperatures of the injector and detector were set at 160 and 200 °C, respectively. For citronellol and geraniol concentration determination, linalool was used as internal standard. Six standard solutions of linalool ranging from 1 μmol mL^{-1} to 40 μmol mL^{-1} were prepared to get the calibration curve. Samples were diluted to the appropriate level to fall within the calibration curve. The product was identified by GC–MS.

2.4. Experimental design, analysis and optimization by response surface methodology

The transesterification reaction was optimized using the Central Composite Design (CCD) and Response Surface Methodology (RSM). The five-level-four-factor CCD was employed in this study, requiring 30 experiments. The reaction temperature (30–70 °C), reaction time (2–10 h), the CPL amount (2–15% based on terpene alcohol weight) and the substrate molar ratio (1:1–3:1 of vinyl octanoate to citronellol) were selected as the important variables for optimization. To avoid bias, 30 runs were performed in a totally random order. The yield of citronellyl ester was taken as the response variable. The design of the employed experiments was presented in Table 1.

Once the experiments were performed, the response variable was fitted to a second-order model in order to correlate the response variable to the independent variable. The general form of the second-order polynomial equation was as follows:

$$Y = \beta k0 + \sum_{i=1}^{4} \beta kiXi + \sum_{i=1}^{4} \beta iiX_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta kijXiXj \quad (1)$$

where Y was the response (dependent variables), β_{k0} was the constant coefficient, β_{ki}, β_{kii} and β_{kij} represented the coefficient for the linear, quadratic and interaction effect, respectively, and X_i and X_j were the independent variables. The coefficients of the response function and their statistical significance were evaluated using Design-Expert 7 software. Contour plots were obtained from the fitted model by keeping the independent variables at a constant value while changing the other two variables.

Table 1
Central composite rotatable second-order design, experimental data for five-level-four-factors response surface analysis.

Run order	Blocks	X_1 (temperature)	X_2 (time)	X_4 (vinyl octanoate to citronellol molar ratio)	X_3 (enzyme concentration)	Yield (%)
1	1	50	6.0	2.00	8.50	85
2	1	40	4.0	1.50	5.25	40.7
3	1	50	6.0	2.00	8.50	86
4	1	50	6.0	2.00	8.50	84.7
5	1	50	2.0	2.00	8.50	30
6	1	60	4.0	1.50	11.75	71.6
7	1	50	6.0	2.00	2.00	55.1
8	1	70	6.0	2.00	8.50	68.9
9	1	60	4.0	2.50	5.25	55.8
10	1	50	6.0	2.00	15.00	93
11	1	60	8.0	2.50	5.25	74.5
12	1	40	4.0	1.50	11.75	62.2
13	1	50	6.0	2.00	8.50	85.4
14	1	60	4.0	1.50	5.25	53.5
15	1	60	8.0	2.50	11.75	72.6
16	1	50	10.0	2.00	8.50	85.6
17	1	40	8.0	2.50	11.75	87.6
18	1	40	4.0	2.50	5.25	42.8
19	1	40	8.0	1.50	11.75	84.8
20	1	50	6.0	2.00	8.50	85
21	1	50	6.0	2.00	8.50	84.5
22	1	40	8.0	2.50	5.25	74.2
23	1	60	4.0	2.50	11.75	66.8
24	1	30	6.0	2.00	8.50	37.9
25	1	50	6.0	3.00	8.50	73.5
26	1	60	8.0	1.50	11.75	79.4
27	1	60	8.0	1.50	5.25	77.5
28	1	40	4.0	2.50	11.75	60.4
29	1	50	6.0	1.00	8.50	83.4
30	1	40	8.0	1.50	5.25	74.5

3. Results and discussion

3.1. Comparison of different terpene alcohols, acyl donors and solvents

The reaction capacities of different terpene alcohols (geraniol, citronellol and linalool) with the vinyl acetate were compared by determining the yield of terpene acetate. After 8 h reaction, the yields of geranyl acetate and citronellyl acetate were 45% and 48% respectively, but no linalyl acetate was detected. This result was similar to the previous studies [19,20]. It could be concluded that the tertiary alcohol (such as linalool) might not be a suitable acyl acceptor for the transesterification with this type of lipase (CPL).

Depending on the nature of the substrates, lipases can catalyze different synthesis reactions: direct esterification and transesterification. Three types of acyl donors (fatty acid, vinyl ester and fatty acid ester) were tested for the synthesis of citronellyl esters. The reaction using fatty acid as acyl donor was a direct esterification whereas the use of vinyl ester or fatty acid ester led to transesterification reaction. The mechanism of CPL catalyzed reaction occurred via a covalently linked acyl-enzyme intermediate, which deacylated through the nucleophilic attack of citronellol or geraniol. This synthetic pathway could be regarded as a kinetically controlled process, where both the rapid accumulation of the acyl-enzyme intermediate and the preferential nucleophilic attack by the alcohol were essential. As shown in Fig. 1, vinyl ester gave the highest yield (e.g. the yield of citronellol caprylate could reach up to 95%), followed by fatty acid and the fatty acid ester reacted slowly. When vinyl esters such as vinyl octanoate was used as activated acid acyl donor, the transesterification reaction could be accelerated because the vinyl alcohol released in the degradation of the vinyl ester tautomerized to acetaldehyde, which could not act as a substrate for the lipase, and thus made the reaction processed irreversibly. When fatty acids used as the acyl donor, an increase in the yield with increasing chain length from C2 to C8 was observed. Further increase in the chain length of the acid up to C14 led to a gradual decrease of yield. In case of fatty acid esters, CPL did not show high activity to acetic acid ester series. As vinyl octanoate showed a good yield of terpene ester, it was used as the acyl donor in the following study.

Selection of proper organic solvent is of significance to lipase-catalyzed reaction in organic solvent. Various solvents with different log P value were investigated for CPL-catalyzed synthesis of terpene esters. As shown in Fig. 2, the highest yield (about 95%) was attained when hexane or cyclohexane was used as the reaction media. The yield also reached to about 90% in the hep-

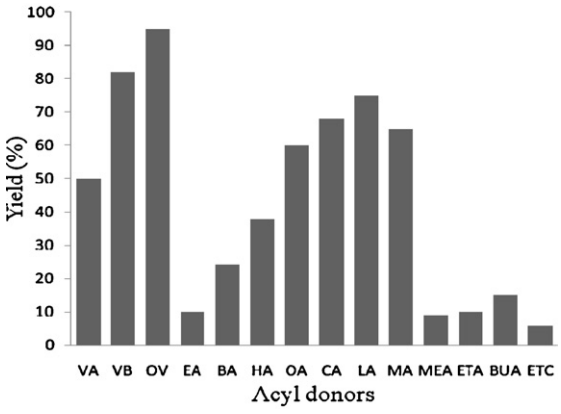

Fig. 1. Effect of different acyl donors on citronellyl ester synthesis. Experimental conditions: 0.003 mol citronellol and acyl donor were dissolved in 4.0 mL of hexane, followed by addition of 50 mg CPL. The reaction mixture was stirred at 200 rpm and 45 °C for 8 h. A control without enzyme was carried out under the same condition. Experiments were carried out in triplicates. VA: vinyl acetate, VB: vinyl butyrate, OV: octanoic acid vinyl ester, EA: ethanoic acid, BA: butyric acid, HA: hexanoic acid, OA: octanoic acid, CA: capric acid, LA: lauric acid, MEA: methyl acetate, ETA: ethyl actate, BUA: butyl acetate, and ETC: ethylcaprylate.

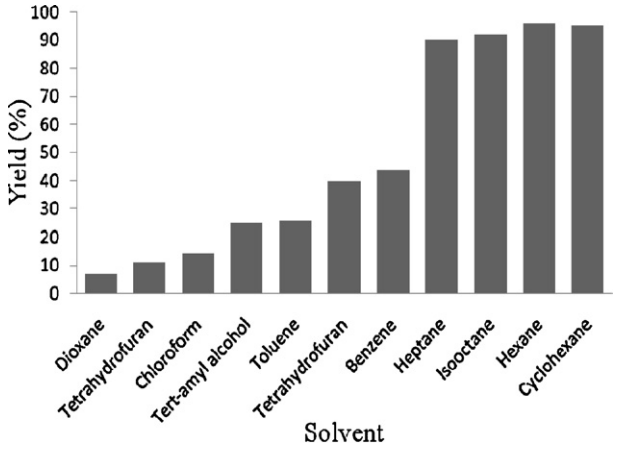

Fig. 2. Effect of different solvents on citronellyl ester synthesis. Experimental conditions: 0.003 mol terpene alcohol and vinyl octanoate were dissolved in 4.0 mL of solvent, followed by addition of 50 mg CPL. The reaction mixture was stirred at 200 rpm and 45 °C for 8 h.

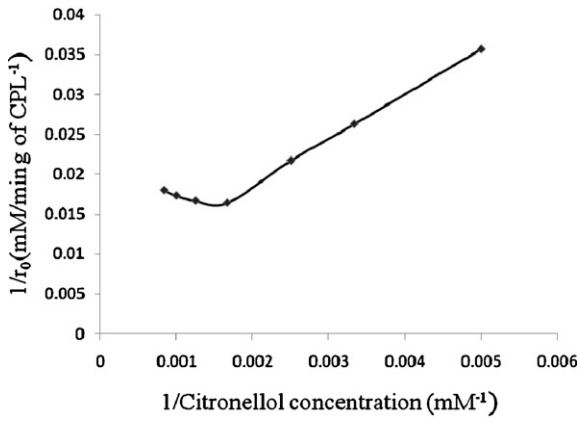

Fig. 3. Lineweaver–Burk plot of reciprocal initial reaction rate versus reciprocal citronellol concentrations at fixed vinyl octanoate concentration in *n*-hexane. Experiments were conducted by using 50 mg CPL with vinyl octanoate fixed at concentration of 0.6 mol/L and the total volume was made up to 5 mL with *n*-hexane. Citronellol amount was varied from 0.2 to 1.0 mol/L.

tane or isooctane system. However, in contrary to previous report [4], the solvents with log *P* between 2.0 and 3.0 only gave moderate yield, with toluene only gave 26% of yield. As far as the yield was concerned, hexane was the optimum media for CPL-catalyzed synthesis of terpene esters.

3.2. Response surface methodology for the optimization of the process variables

3.2.1. Development of regression model

RSM was used to understand and optimize the relationship between the important reaction parameters in the CPL-catalyzed synthesis of citronellyl caprylate in organic media. The statistical combination of design variables with response (yield of citronellyl caprylate) was presented in Table 1.

The model *F*-value of 22.36 implied that the model was significant. The *P*-value was less than 0.0001, i.e., there was a 0.1% chance that this error was caused by noise. This implied a very high significance of the regression model. The goodness of fit of the model was checked by the determination coefficient (R^2). In this case, the value of R^2 (=0.9511) indicated that the model did not explain only 4.89% of the total variations. The value of adjusted determination coefficient (Adj R^2 = 0.9055) was also high to get a high significance of the model. A high value of correlation coefficient *R* (=0.95) indicated a close agreement between predicted value and actual value of response. At the same time a relatively lower value of coefficient of variation (CV = 7.30%) indicated a better precision and reliability of experiments carried out.

The application of response surface methodology resulted in the following regression equation in the coded unit:

$$Y = 85.10 + 3.60X_1 + 11.77X_2 + 1.22X_3 - 6.99X_4 + 7.67X_1^2 - 6.57X_2^2 - 1.41X_3^2 - 2.51X_4^2 + 3.67X_1X_2 - 0.94X_1X_3 - 2.11X_1X_4 - 0.32X_2X_3 - 2.78X_2X_4 - 0.73X_3X_4$$

where *Y* was the citronellyl caprylate yield (%), X_1 was the coded value of the temperature, X_2 was the coded value of the time, X_3 was the coded value of the enzyme concentration, and X_4 was the coded value of the molar ratio of vinyl octanoate to citronellol. The concentration of citronellol is fixed at 0.75 mmol/mL, the concentration of vinyl octanoate is changed when changing the substrate molar ratio.

3.2.2. Kinetic analysis of the reaction and optimization of reaction conditions

The main emphasis of this part was to develop a kinetic model, and then optimize the reaction conditions such as temperature, the reaction time, enzyme concentration, molar ratio of vinyl octanoate to citronellol to maximize the yield and reduce the production cost under the most favorable conditions. Hence the effects of concentration of both the reactants on the reaction rate were studied systematically over a wide range. For the determination of initial rates, several mechanisms have been proposed to explain lipase catalyzed reactions, e.g. a sequence of various irreversible consecutive pseudo first orders reactions and ordered Bi–Bi mechanism. However, the generally accepted mechanism is the so called Ping–Pong Bi–Bi mechanism [21]. In the Ping–Pong Bi–Bi mechanism, a product is released between additions of two substrates. Two set of experiments were conducted by using 50 mg CPL with appropriate quantities of citronellol and vinyl octanoate, and the total volume was made up to 5 mL with *n*-hexane. In one set of experiment citronellol amount was varied from 0.2 to 1.0 mol/L at a fixed quantity of vinyl octanoate (0.6 mol/L), and in the other set, the amount of vinyl octanoate was varied from 0.2 to 1.0 mol/L at a fixed quantity of citronellol (0.6 mol/L). From the initial rate measurements, it was observed that the rate increased with increasing concentration of vinyl octanoate, but the inhibition by citronellol appeared when the concentration is greater than 0.6 mol/L. Reciprocal initial reaction rates (v^{-1}) were plotted versus the inverse citronellol concentration (Lineweaver–Burk plot) at fixed vinyl octanoate concentration, and the results are shown in Fig. 3. Citronellol inhibition was confirmed, since at low values of the inverse of its concentration the lines curved upward. There was no evidence of inhibition by vinyl octanoate at any concentrations tested.

These results agree with the assumed Ping–Pong Bi–Bi mechanism with dead end inhibition. For this reaction at high citronellol concentration the lipase may react with it to yield a dead end enzyme–citronellol complex or it may react with vinyl octanoate to yield the effective lipase–vinyl octanoate complex. Then the lipase–vinyl octanoate complex is transferred to an enzyme-acyl intermediate and acetaldehyde is released. This is followed by the interaction of the enzyme–acyl complex with citronellol to form another binary complex, which then yields the ester and free lipase. The final equation of the reaction is given as follows:

$$\frac{V}{V_{max}} = \frac{[A][B]}{K_{i(A)}K_{m(B)} + K_{m(A)}[B] + K_{m(B)}[A] + [A][B]} \quad (2)$$

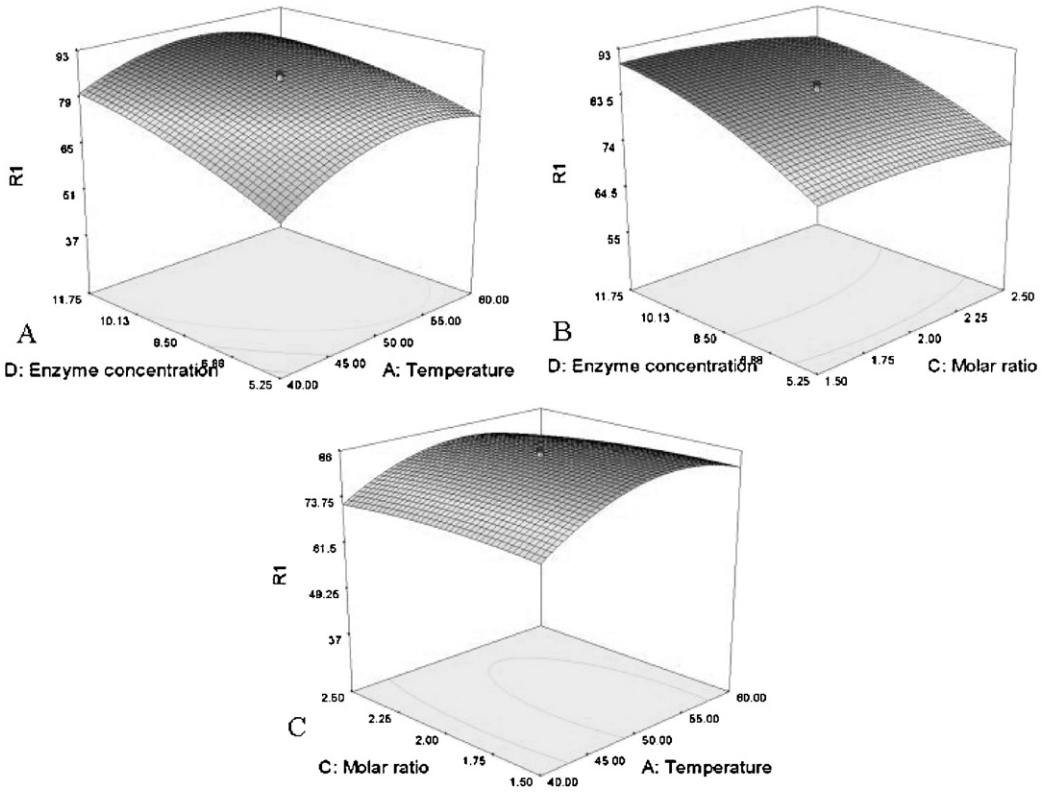

Fig. 4. Contour plot of the combined effect of (A) temperature and enzyme concentration, (B) substrate molar ratio and enzyme concentration, and (C) temperature and substrate molar ratio.

Lineweaver–Burk equation is obtained as follows for which initial rate data and concentration are used.

$$\frac{1}{V_0} = \frac{K_{i(A)}K_{m(B)} + K_{m(A)}[B_0] + K_{m(B)}[A_0] + [A_0][B_0]}{V_{max}[A_0][B_0]} \quad (3)$$

where $[A_0]$ is the initial concentration of citronellol, $[B_0]$ the initial concentration of vinyl octanoate, $K_{m(A)}$ the Michaelis constant of citronellol, $K_{m(B)}$ the Michaelis constant of vinyl octanoate, K_i the inhibition constant of citronellol, V_0 the initial rate of the reaction, V_{max} is the maximum rate of the reaction. The data from initial rate measurements were used for the calculation of the kinetic parameters by multiple regressions, as given below:

- V_{max} = 142.9 mmol/(min/g enzyme)
- $K_{m(A)}$ = 821.7 mmol/(min/g enzyme)
- $K_{m(B)}$ = 30.9 mmol/(min/g enzyme)
- $K_{i(A)}$ = 12.1 mmol/(min/g enzyme)

Based on the calculated kinetics parameters, the affinity of the CPL towards citronellol seems to be smaller than that of vinyl octanoate, since K_{mA} (821.7 mM) was higher than K_{mB} (30.9 mM). These results conform to the Ping–Pong mechanism, which proves that the vinyl octanoate is the first substrate that binds to the lipase.

As the inhibition by citronellol was observed at the concentration above 0.6 mol/L, its concentration was fixed at 0.6 mol/L in the RSM optimization of the reaction conditions. The significance of each coefficient is determined by *P*-values, the *P*-values of X_1, X_2, X_3 and X_4 are <0.0001, 0.0620, 0.0002 and 0.0928. Judged from the *P*-values, temperature and enzyme concentration were the most significant factors ($P < 0.05$).

Temperature plays an important role in the reaction kinetics when enzymes are used as catalyst, an increase in temperature could increase the diffusion coefficients of the substrates migrating to the active sites of enzymes, enhanced the energy of the reaction system and reaction rate [22]. Therefore, the yield and reaction rate increased with the rise in the reaction temperature and reached a maximum at 55 °C. Notable decrease in the yield was observed when the temperature increased from 60 °C to 70 °C as shown in Fig. 4(A). The response surface plot as the interaction of CPL concentration and reaction temperature on citronellyl caprylate synthesis. The typical plots like this found by other workers were dome shape [23,24]. In this work, however, there was a linear increase in yield at one axis, but there was a increase only up to some extent at the other axis. High temperature not only lowered the yield but also reduced the operation stability of CPL (data not shown). These indicated that a critical temperature was involved, up to which synthesis was favored and it was not so after that critical temperature. This phenomenon was in accordance with the report that the CPL could lose its activity at the temperature higher than 60 °C [25].

Another important factor influencing the yield is CPL concentration. The yield was lower at low CPL concentration, but it increased rapidly up to about 90% with CPL loading at the point of 12%, and after that further addition of CPL could not increase the yield obviously as shown in Fig. 4(B). This could be explained by considering that the active sites of the CPL molecules presenting in excess would not be exposed to the substrates and remain inside the bulk of enzyme particles without contributing significantly to the reaction [26].

According to the kinetics of enzyme-catalyzed reaction, for two substrates involved reaction the reaction rate $V = k[A][B]$ in which k was the rate constant, [A], [B] were the concentration of the two substrates. So presence of large amount of substrates generally increased the reaction rate and yield [27]. In other words when a substrate concentration was fixed, the substrate molar ratio (X_4) had significant effect on reaction rate and yield [1], but in this work the yield only changed slightly at the substrate molar ratio ranging from 1:1 to 3:1, high substrate molar ratio (e.g. 1:3.0) even lead to a little decrease in yield (Fig. 4(C)). Explanation for this might be that

the acyl donor (vinyl octanoate) used was an active acyl donor. On one hand, the vinyl alcohol released in the degradation of the vinyl ester tautomerized to acetaldehyde which could not further act as a substrate for the lipase, and thus made the reaction irreversible and accelerated the reaction; on the other hand, acetaldehyde could dissolve in water and most of organic solvents such as hexane. The acetaldehyde produced during the reaction could not volatilize outside the screw-capped vial, which made the acetaldehyde dissolved into the reaction media. The accumulated acetaldehyde was considered to inactive the enzyme by formation of a Schiff base with the lysine residue of enzyme protein [16–18]. Substrate molar ratio had no significant effect, which indicated that synthesis of terpene esters with low substrate molar ratio (1:1) was possible. Such an application would reduce the production cost by decreasing the amount of cosubstrate and facilitating the separation of product.

3.2.3. Attaining optimum conditions

The optimal conditions for the CPL-catalyzed synthesis of citronellyl caprylate were predicted using the optimization function of the Design Expert Software. To reduce the production cost, the optimum reaction conditions for the citronellyl caprylate synthesis were selected as follows: 55 °C, 9% of CPL concentration based on substrate weight and equimolar ratio of citronellol and vinyl octanoate. Under these conditions the yield of more than 99% could be achieved after 8 h.

3.3. Operational stability of CPL

Although CPL-catalyzed synthesis of terpene ester in organic media was fulfilled, this technique suffered from a critical drawback, i.e., poor operational stability of CPL. As shown in Fig. 5, the CPL retained only 51% of the original activity after three batches.

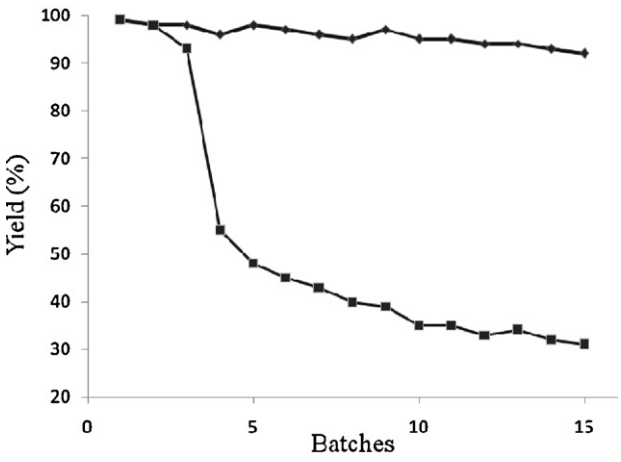

Fig. 5. Reusability of CPL in organic solvent. IL coated CPL (♦) and CPL without any pretreatment (■). The reaction was conducted at 55 °C with equimolar ratio of citronellol and vinyl octanoate in hexane. After 8 h, the CPL was filtered and washed with hexane fully, and then repeated the reaction with fresh substrates.

Foglia and Villeneuve [12] also reported the similar phenomenon when using CPL to catalyze the synthesis of structured triacylglycerols. The poor operational stability of CPL might be due to the following reasons: (1) the lipase protein might scale off the "naturally immobilized carriers" because of the disintegration of these unidentified carriers in organic solvent; (2) the CPL lost inevitably during filtration every batch; (3) the organic solvent might also strip off the essential water around the CPL molecule, which thereby affected the active conformation of CPL and led to activity loss; (4) the acetaldehyde produced during the synthesis reaction inacti-

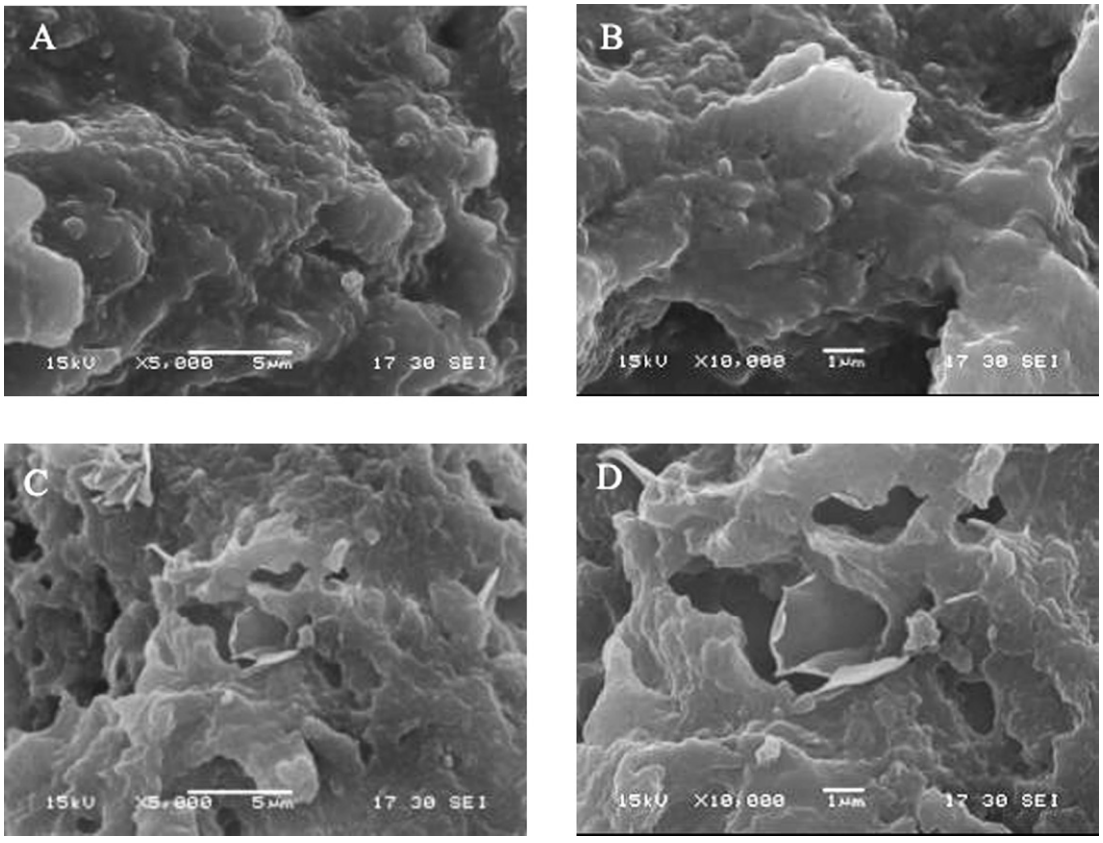

Fig. 6. (A) Scanning electron microscope (SEM) image of the ionic liquid coated CPL (5000× magnification). (B) SEM image of the ionic liquid coated CPL (10,000× magnification). (C) SEM image of the CPL (5000× magnification). (D) SEM image of the CPL (10,000× magnification).

vated the activity of CPL to some degree. As reported by Brigitte Berger and Ganapati D. Yadav in their work [1,16], we also found acetaldehyde showed negative effect on CPL activity. The yield decreased with the increase amount of acetaldehyde initially added to the reaction medium. When 1.5 mmol acetaldehyde was added, the yield sharply decreased to 17% (date not shown). This suggested that CPL should be deactivated in the presence of acetaldehyde. In this work we attempted to use ionic liquids to overcome the negative effects of acetaldehyde and organic solvent and to improve the operational stability of lipase.

Room temperature ionic liquids are composed of ions exhibit certain properties which make them attractive media for performing green catalytic reactions. They have essentially no vapour pressure and are thermally robust compared to water. Polarity and hydrophilicity/hydrophobicity can be tuned by a suitable combination of caution and anion, which has earned them the accolade 'designer solvents'. The use of ionic liquids as reaction media for biotransformations has several potential benefits compared to conventional organic solvents, e.g. higher operational stabilities and activities [28]. Five different ionic liquids (1-butyl-3-methylimidazolium [bmim], 1-hexyl-3-methylimidazolium [hmim], 1-methyl-3-octylimidazolium [omim], 1-decyl-3-methylimidazolium [demin] associated with the same anion (hexafluorophosphate, [PF_6]) and 1-butyl-3-methylimidazolium tetrafluoroborate [bmim] BF_4) based on alkyl imidazolium cautions were investigated as the reaction media for CPL-catalyzed synthesis of terpene ester. It was found that the CPL showed the highest activity in [PF_6] based anion IL with yield up to 99% in [bmim] PF_6, and the lowest yield (40%) was observed in [bmim] BF_4 (data not shown). Notwithstanding the advantages of ionic liquids as reaction media for biocatalytic processes, their relative high viscosity and price made them yet not to be widely applied. Considering this, we developed a new method for reducing these defects by coating the naturally immobilized CPL particles with [bmim] PF_6 and used the resulting IL-coated CPL to catalyze the synthesis of terpene ester. The result showed that the IL-coated CPL presented high catalytic activity, and the yield could attain nearly 100% in less than 8 h. The operational stability of IL-coated CPL was 5 times more than that of uncoated CPL. About 90% of the original activity could remain after 15 cycles use (Fig. 5).

This IL should be regarded as a protective shell rather than a reaction medium as shown in Fig. 6. The SEM pictures clearly showed that the ionic liquid formed a protective shell on the surface of the CPL, and compared to the uncoated CPL the structure of the IL coated CPL seemed to be more compact after 8 h reaction. On one hand, as the acetaldehyde had poor solubility in the IL ([bmim] PF_6), the IL presented on the CPL was considered to preserve active enzymes in non-aqueous environment by avoiding the denaturation caused by direct interaction with the acetaldehyde which could react with the amino group of the protein to form Schiff base, as reported by Hamsaveni [29], the Schiff base had negative effect on the reactive of the enzyme. On the other hand, the water-immiscible ILs such as [bmim] PF_6 formed a strong ionic matrix which retained CPL molecules in an adequate microenvironment, resulting in a supramolecular net to keep the protein conformation active, which could be related to prevention of water stripping caused by hexane and the preservation of the essential water shell, and was able to stabilize the enzyme through the formation of a flexible and more compact three-dimensional structure.

4. Conclusion

The CPL was used as an effective biocatalyst to catalyze the synthesis of citronellyl caprylate successfully in this work. This method reported was mild and "clean" as compared with chemical methods. Nearly 100% yield and purity were obtained after 8 h reaction at equimolar ratio of citronellol and vinyl octanoate, 9% (w/w) of CPL loading and 55 °C. The operational stability of CPL was greatly improved by coating the enzyme particles with IL, and at least 15 batches could be used. By making suitable modifications in the optimized reaction conditions obtained in the present study, various other monoesters of different flavors could be produced effectively. CPL was proved to be a potential and promising lipase for the synthesis of different terpene esters.

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