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Experimental and optimization for kinetic resolution of 1-(4-(trifluoromethyl)phenyl)ethanol enantiomers by lipase-catalyzed transesterification in organic phase

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Abstract
Kinetic resolution of 1-(4-(trifluoromethyl)phenyl)ethanol (TFMP) enantiomers was achieved through lipase-catalyzed transesterification in organic solvents. Lipase PS from *Pseudomonas cepacia* was selected as the best biological catalyst, and vinyl acetate was used as the acyl donor for the transesterification in isooctane. The effects of temperature, enzyme dosage, substrate ratio and time on the reaction were investigated. Response surface methodology was introduced as the tool for process optimization and the optimized conditions were obtained. The experimental results under the optimized conditions involving the temperature of 46 °C, substrate ratio of 1:12, enzyme dosage of 15 mg and time of 104 min, show that TFMP enantiomers were resolved with the enantiomeric excess of the remaining substrate (ee_s) higher than 99.0% and the conversion (c) of 50.3%, which indicates an efficient kinetic resolution process.

Keywords Kinetic resolution · Transesterification · Lipase PS · Response surface methodology · 1-(4-(Trifluoromethyl)phenyl)ethanol

Introduction

The preparation of homochiral secondary alcohols is of significant importance in chemical, pharmaceutical and related fields, because the homochiral secondary alcohols provide building blocks for a wide range of biologically active compounds [1]. Methods to access homochiral secondary alcohols can be divided into three categories, synthesis starting from chiral pools, asymmetric synthesis and resolution of the racemate [2, 3]. Despite the remarkable progress in asymmetric synthesis, kinetic resolution of racemates is still the dominant method for production of homochiral

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secondary alcohols in industry, where the two enantiomers are resolved based on their different reaction kinetics in chiral entity [4]. The chiral entity is the key factor in kinetic resolution, it can be provided by a biocatalyst like enzyme and microorganism or a chemocatalyst like chiral acid, chiral base and chiral metal complex [5, 6]. Much attention has been paid to the chiral synthesis by chemocatalyst, however, biocatalyst has already gathered significant interest in kinetic resolution [7–9].

Use of a biocatalyst allows the process to be operated under mild conditions, and the inherent high regio- and enantioselectivity improves the atomic economy [10, 11]. Furthermore, the biocatalyst is biodegradable. Therefore, kinetic resolution with a biocatalyst, which better conforms to the concept of modern green chemical industry, is particularly advantageous [12, 13]. Among numerous biocatalysts, lipase has been found to be interesting in kinetic resolutions for production of optically pure compounds [14]. Lipases are hydrolases (E.C.3.1.1.3) and they can catalyze the hydrolysis of triglycerides into glycerol and fatty acids [15, 16]. Lipases with excellent catalytic properties have been well studied as catalysts because of their unique physicochemical behavior [17–19]. Enzymes are remarkably effective in the versatile reactions, such as hydrolysis [20–22], esterification [19, 23], transesterification [24], alcoholysis [25], and C–C bond formation [18]. Enzymes have been recently used as a potential biocatalyst in a large number of biotechnological sciences [26], more specifically, these include dairy products, detergents [18], pharmaceuticals [27, 28], chemicals [29, 30], agriculture products [18], and oil chemistry [30–33]. Enzymes act as a good catalyst, therefore, its production and utilization may be a better alternative of chemical catalysts.

The lipase-catalyzed kinetic resolutions are usually through a stereoselective reaction of nucleophiles with esters or their derivatives, such as a stereoselective transesterification reaction. Lipase has an active center of serine-histidine-aspartate catalytic triad [34], which is usually covered by a flexible region of the enzyme molecule, often called the lid. Interaction with a hydrophobic phase can cause opening of the lid to make the active site accessible [35]. Therefore, a lipase expresses higher catalytic activity at an organic–aqueous interface than in aqueous solution and the phenomenon is called interfacial activation [36]. The use of a low boiling point organic solvent in lipase-catalyzed transesterification can significantly increase the solubility of the substrates in reaction media and facilitate the recovery of the product with better overall yield [37, 38]. Furthermore, the use of lipase in an organic solvent offer several advantages, for example it minimizes the substrate or product inhibition, the possibility of denaturation and it also make the immobilization of enzymes not always required [39]. To promote the equilibrium of the acylation reaction shifting to the desired direction and inhibit the reverse reaction, the selected acyl donor is required, such as enol esters, whose leaving group is an enol that immediately suffer from a tautomeric reaction thereby promoting the reaction tends to be complete [40]. Thus, in the lipase-catalyzed kinetic resolution of racemic alcohols, the presence of a suitable lipase and acyl donor in an appropriate organic solvent, as well as the optimum temperature, enzyme dosage, substrate ratio and reaction time are all the important factors for biosynthesis of an optically pure alcohol product [41, 42]. The process optimization is therefore is very necessary in investigation of the above process. Response surface method (RSM) is a powerful

tool that has been used to evaluate the interactions between multiple process factors and simulate the quantitative relation between the response values and the factors, which can further be used for process optimization [43].

1-[4-(Trifluoromethyl)phenyl] ethanol (TFMP) is an important intermediate for the synthesis of AIDS (acquired immune deficiency syndrome) drugs, and (*R*)-TFMP is an important intermediate for the synthesis of AD101 (SCH-350581), a chemokine CCR5 antagonist, that blocks the entry of HIV-1 into cells [44–46]. Chen et al. [44] achieved the chemical synthesis of (*R*)-TFMP by asymmetric reduction of 4-(trifluoromethyl)acetophenone over a chiral chemical catalyst of oxazaborolidine, however the expensive chiral catalyst and the potential risk of environmental pollution limit its application prospect. The method is improved through employing a recombinant whole cell to catalyze the asymmetric reduction, but the method is still restricted by the difficulty in process control [44]. Compared with the above, lipase-catalyzed kinetic resolution is an attractive method to prepare (*R*)-TFMP. Lipase-catalyzed kinetic resolution allows the resolution of TFMP enantiomers with high optical purity. The process can be operated under mild conditions and control of the process is much convenient.

Herein, we report the kinetic resolution of racemic TFMP through lipase-catalyzed enantioselective transesterification in the organic media. Lipase used this work is selected from the commercially available lipases and the effects of important process parameters such as temperature, substrate ratio, enzyme dosage and time on the resolution efficiency were investigated. RSM is further employed for process optimization to achieve a high efficiency of kinetic resolution.

Experimental section

Lipase and reagents

All lipases used in this work were commercially available and used in the experiments without further treatment. The origin of the lipases and other related information is shown in Table 1.

(*R,S*)-1-[4-(Trifluoromethyl)phenyl]ethanol (purity > 98% +) and vinyl acetate (purity > 99% +) was purchased from Adamas reagent Co., Ltd. (Shanghai, China). The *n*-hexane and isopropanol used for HPLC detection were chromatographic grade, while the other reagents were analytical grade and were purchased from different companies. All reagents were applied to the reaction without further treatment.

HPLC analysis

The quantification of TFMP enantiomers was performed by a Waters e2695 high performance liquid chromatography (HPLC) consisting of a Waters e2695 separation unit, a 2489 UV–visible detector. Daicel Chiralcel IG column (250 mm × 4.6 mm ID, Tokyo, Japan) was used. The mobile phase was composed of n-hexane/anhydrous

Table 1 The properties of the enzymes used in this paper

Enzyme	Source	Supplier	Status	Optimum T and pH	Activity
Lipase AYS	*Candida rugosa*	Amano Pharmaceutical Co. Ltd. (Nagoya, Japan)	White lyophilized poeder	T = 40 °C pH 7	30,000[a]
Lipase AK	*Pesedomonas fluorescens*	Amano Pharmaceutical Co. Ltd. (Nagoya, Japan)	Light yellow lyophilized powder	T = 60–70 °C pH 4–10	20,000[a]
Lipase PS	*Pseudomonas cepacia*	Amano Pharmaceutical Co. Ltd. (Nagoya, Japan)	white Lyophilized powder	T = 50 °C pH 7	30,000[a]
Lipozyme CALB	*Candida antarctica B*	Novozymes Biopharma DK A/S (Denmark)	Transparent viscous liquid	T = 30–60 °C pH 5–9	5000[b]
Novocor ADL	*Candida antarctica A*	Novozymes Biopharma DK A/S (Denmark)	Brown liquid	T = 30–60 °C pH 5–9	6000[b]
Lipozyme RM IM	*Rhizomucor miehei*	Gao Ruisen Technology Co., Ltd (Beijing, China)	Immobilized	T = 50 °C pH 7	250[c]
Lipozyme TL IM	*Candida cylindracea*	Gao Ruisen Technology Co., Ltd (Beijing, China)	Immobilized	T = 50–75 °C pH 6–8	250[c]

[a]PLU g^{-1} = propyl laurate unit

[b]LU g^{-1} = lipase unit

[c]IUN g^{-1} = interesterification unit

ethanol 99:1 (v/v). The flow rate was maintained at 1 mL min^{-1}. Injection volume of each sample was 10 μL; The column temperature was set at 25 °C. The wavelength is 210 nm. The enantiomer excess of the substrate (ee_s), the total conversion (c), the conversions of (R)-enantiomer (c_R) and the conversions of (S)-enantiomer (c_S) were calculated by Eqs. 1, 2, 3 and 4:

$$ee_s = \frac{[R] - [S]}{[R]_o + [S]_o} \times 100\%, \tag{1}$$

$$c = \left(1 - \frac{[R] + [S]}{[R]_0 + [S]_0}\right) \times 100\%, \tag{2}$$

$$c_R = \frac{[R]_0 - [R]}{[R]_0} \times 100\%, \tag{3}$$

$$c_S = \frac{[S]_0 - [S]}{[S]_0} \times 100\%, \tag{4}$$

Here $[R]$ and $[S]$ are the concentrations of (S)-TFMP and (R)-TFMP in reaction mixture, respectively; $[R]_0$, $[S]_0$ is initial amount of (S)-TFMP and (R)-TFMP, respectively.

Lipase-catalyzed transesterification of TFMP

The experiments were performed in a 25 mL glass tube with a spiral seal. A typical experimental procedure was as follows: the reactants of TFMP racemate (5 mmol L^{-1}) and vinyl acetate (30 mmol L^{-1}) were dissolved in 25 mL of isooctane to form the raw material solution. Then, 2 mL of the raw material solution was added into the glass tube. The solution was stirred (500 rpm min^{-1}) and heated by a thermostatic stirrer (IKA RCT Basic, Germany). After reaching the temperature of 45 °C, 15 mg of lipase was added. After the reaction was completed, the reaction mixture was poured out and filtered to obtain the sample HPLC analysis. Lipases, acyl donors and organic solvents were selected through screening experiments. Effects of conditions including temperature (25–65 °C), enzyme dosage (5–30 mg), molar ratio of TFMP to vinyl acetate (1:1–1:48) and reaction time (20–170 min) were investigated and optimized. The reaction is illustrated in Fig. 1. In this work, the enzyme dosage refers to the mass of various lipases including the powder of lyophilized enzyme, the solution of enzyme and the immobilized lipase (including the support), all of which are weighed and added into the reaction medium.

Kinetic study

Experiments on lipase-catalyzed transesterification between racemic TFMP and vinyl acetate were carried out at different TFMP concentrations and a constant concentration

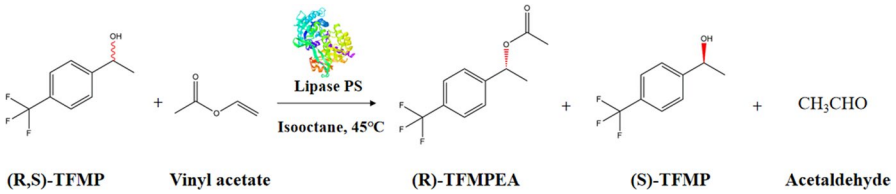

Fig. 1 Lipase-catalyzed transesterification of TFMP in isooctane

of vinyl acetate (90 mmol L^{-1}), using lipase PS as the catalyst. The sample was drawn at the certain stages of reaction for analysis of the concentration of (*R*)-TFMP. The TFMP concentration–time curves were recorded (control the conversion of (*R*)-TFMP less than 20%) and the initial reaction rate V_0 was determined by making the slope of the concentration–time curves in the initial reaction stage.

Experimental design and data analysis

Generally, lipase-catalyzed transesterification of TFMP is influenced by a series of factors, such as lipase, organic solvent, acyl donor, enzyme dosage, substrate molar ratio, temperature, and reaction time. On the basis of single factor investigation, Response surface methodology (RSM) method and Box-Behnken design were used to optimize the process.

The key to response surface optimization is the selection of experimental points. Therefore, the reasonable value range of the main influencing factors has been determined through single factor experiment. Then response surface analysis was used to optimize the lipase-catalyzed resolution conditions. Show in Table 2, the four independent variables in this work were temperature (A, 25–65 °C), substrate ratio (B, 1–24), enzyme dosage (C, 5–25 mg) and time (D, 60–160 min).

$$c = \alpha_0 + \sum_{i=1}^{k} \alpha_i x_i + \sum_{i=1} \sum_{j=i+1}^{k} \alpha_{ij} x_i x_j + \sum_{i=1}^{k} \alpha_{ii} x_i^2, \tag{5}$$

$$ee_s = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1} \sum_{j=i+1}^{k} b_{ij} x_i x_j + \sum_{i=1}^{k} b_{ii} x_i^2, \tag{6}$$

Table 2 Independent variables and their ranges

Factors	Level		
	− 1	0	1
A-temperature (°C)	25	45	65
B-substrate molar ratio	1	12.5	24
C-dosage of enzyme (mg)	5	15	25
D-time (min)	60	110	160

Here, c and ee_s represent conversion and enantiomeric excess of the remaining substrate, respectively; α_0 and b_0 are the constant coefficient; α_i and b_i, α_{ij} and b_{ij}, α_{ii} and b_{ii} are the linear coefficient, squared coefficient, and cross-product coefficient, respectively; k is the number of factors; x_i and x_j are the independent variable. All the coefficients in Eqs. 5 and 6 were calculated by analysis of variance (ANOVA). Design Expert (version 10.0.1) was employed to acquire all coefficient of model and optimize the multiple responses.

Results and discussion

Screening of lipase

Selection of a suitable lipase is very important for the lipase-catalyzed kinetic resolution. Nine commercially available lipases are tested and the results are shown in Table 3. It is found that different lipases exhibit large difference in catalytic activity (indicated by the substrate conversion, c) and selectivity (indicated by the enantiomeric excess of the remaining substrate, ee_s). Several lipases including lipase AK, lipase PS and Novocor ADL have good catalytic activity towards the substrate, the high conversion of 42.6% and 43.8% are achieved with lipase AK and lipase PS, among which lipase PS shows the best enantioselectivity with ee_s of 74.8% and c of 43.8%. Therefore, lipase PS was selected for the further investigation.

Screening of organic solvents

Lipase-catalysis in organic solvents broadens the use of biocatalyst, which is conventionally used in the aqueous media. It is also founded that the catalytic efficiency of the lipase is largely influenced by the solvent [47]. In order to select the most suitable solvent, the effect of organic solvents was investigated and the results are shown in Table 4. It is observed that the activity (indicated by c) was largely influenced by organic solvent and the influence largely depended on the polarity organic solvent [48–51]. Log$P$ is used to evaluate the solvent polarity in general, and it would be increased with increasing the hydrophobicity of the solvent [52]. Lipase PS showed

Table 3 The resolution efficiency of different lipases

Lipase	ee_s (%)	c (%)
Lipase AYS	1.3	3.5
Lipase AK	69.0	42.6
Lipase PS	74.8	43.8
Lipozyme CALB	55.8	37.3
Novocor ADL	59.9	42.3
Lipozyme RM IM	33.1	29.5
Lipozyme TL IM	24.2	20.0

Conditions: 5 mmol L^{-1} (*R,S*)-TFMP; 30 mmol L^{-1} vinyl acetate; 15 mg lipase; 2 mL isooctane; $T = 45$ °C; $t = 720$ min

Table 4 Effects of organic solvents

Organic solvent	log*P*	ee_s (%)	*c* (%)
Isooctane	4.37	81.8	46.6
n-hexane	3.76	74.4	47.6
Toluene	2.5	8.04	24.94
Iso-propyl ether	1.90	10.55	11.37
1,2-Dichlororthane	1.46	3.47	4.94
MTBE	1.40	2.11	5.84
Dichloromethane	0.60	14.92	13.93

Conditions: 5 mmol L^{-1} (*R,S*)-TFMP; 30 mmol L^{-1} vinyl acetate; 15 mg lipase PS; 2 mL organic solvent; $T = 45$ °C; $t = 720$ min

the highest activity and enantioselectivity when n-hexane and isooctane were used as solvents. The catalytic activity of lipase PS is reduced with the increase of the polarity of the solvent. The possible reason is that the hydration layer on the surface of the lipase molecules is necessary for the lipase to maintain its activated conformation [53]. Low polarity of the solvent well protects the hydration layer, but a polar solvent will enter and damage the hydration layer [54]. The highest *c* of 46.6% and ee_s of 81.8% was achieved with isooctane. Therefore, isooctane with log*P* of 4.3 was selected as organic solvent in this work.

Screening of acyl donors

In the lipase-catalyzed transesterification of TFMP racemate, the (*R*)-TFMP selectively reacts with the acyl donor to form the (*R*)-TFMP ester with high *ee* value and leaving (*S*)-TFMP as the enantiomerically pure unreacted enantiomer. The selection of acyl donor is therefore important. As is shown in Table 5, some acyl donors are tested. With the enol esters (vinyl acetate, vinyl propionate, isopropenyl acetate and vinyl butyrate), the resolution efficiency is significantly enhanced than that with other donors. This is because the leaving group of these donors undergoes a keto–enol tautomerization to yield the corresponding carbonyl compound (such as acetaldehyde), thereby preventing the reverse reaction and driving the reaction to completion. Based on the results in Table 5, vinyl acetate was selected.

Table 5 Effects of acyl donors

Organic solvent	c_R (%)	c_s (%)	ee_s (%)	*c* (%)
Vinyl acetate	90.20	0.22	82.04	45.19
Ethyl acetate	48.81	1.00	31.84	24.90
Vinyl propionate	86.08	1.18	75.31	43.63
Isopropenyl acetate	89.72	2.90	80.85	46.31
Vinyl butyrate	87.70	0.01	78.10	43.85
Isobutyl acetate	39.42	0.76	24.19	20.09

Conditions: 5 mmol L^{-1} (*R,S*)-TFMP; 30 mmol L^{-1} acyl donor; 15 mg lipase PS; 2 mL isooctane; $T = 45$ °C; $t = 720$ min

Effect of temperature

Since enzyme is a protein in chemical nature, temperature has a significant influence on the catalytic activity of the lipase [55, 56]. The effects of temperature on catalytic activity and enantioselectivity of lipase PS in the present lipase-catalyzed transesterification reaction system were studied in the range from 25 to 65 °C. As can be seen from Fig. 2, with the increase of temperature, the c and ee_s increase steadily. The conversion reaches its maximum at 45 °C and can maintain the maximum in the range from 45 to 60 °C, indicating that lipase PS has good thermal stability.

Effect of enzyme dosage

The enzyme dosage is also an important factor affecting the lipase-catalyzed resolution efficiency. As shown in Fig. 3, when the amount of enzyme increases from 5 to 15 mg, both c and ee_s gradually increase to the optimal value, where c of about 50.0% and ee_s higher than 99.0% are achieved. The powder of lipase PS was well dispersed in the reaction system. Furthermore, the substrates are well dissolved in the organic solvent reaction system. Therefore, diffusion limitations for the reaction system in this work is minimized. However, with the further increase of enzyme dosage, it can be observed that c and ee_s have been stable near the optimal value without change. This phenomenon can be explained by the fact that the dispersion of the powder of lipase PS becomes difficult and the contact between the enzyme and the substrate is not significantly increased when the enzyme dosage is high than 15 mg, thus the further increase in enzyme dosage don't improve the conversion. The other possible reason is that the enzyme may experience protein aggregation and it made the catalytic center less accessible [57]

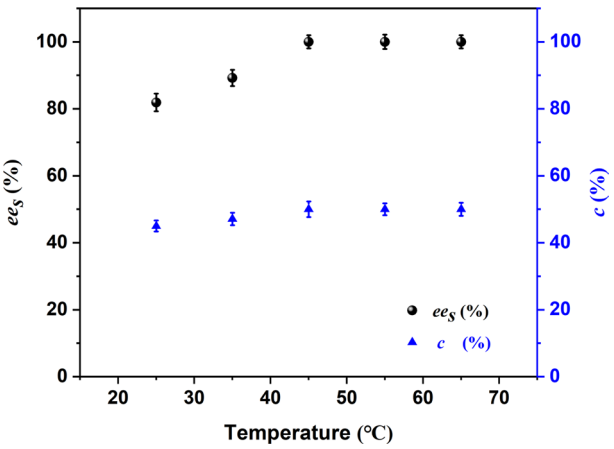

Fig. 2 Effect of temperature on the resolution of TFMP enantiomers. Conditions: 5 mmol L^{-1} (*R,S*)-TFMP; 60 mmol L^{-1} vinyl acetate; 15 mg lipase PS; 2 mL isooctane; $t = 720$ min

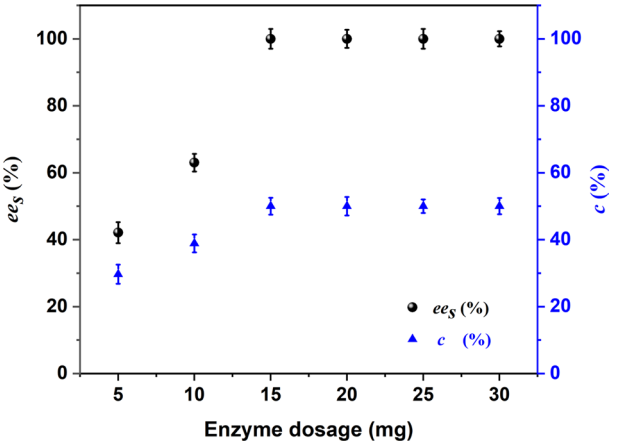

Fig. 3 Effect of enzyme dosage on the resolution of TFMP enantiomers. Conditions: 5 mmol L^{-1} (*R,S*)-TFMP; 60 mmol L^{-1} vinyl acetate; 2 mL isooctane; $T = 45$ °C; $t = 720$ min

Effect of substrate ratio

Substrate ratio (molar ratio of vinyl acetate to TFMP) is also an important factor affecting the resolution efficiency. As is shown in Fig. 4, when the substrate ratio increased from 1:1 to 1:12, the c and ee_s increase rapidly to a high value. When the substrate ratio is further increase from 1:12, both c and ee_s reach a platform and remain basically unchanged. The possible reason is that when the addition of vinyl acetate is small, a large amount of catalytic active sites is not occupied and the increasing in addition of vinyl acetate can accelerate the reaction rate and promote a forward shift of the reaction equilibrium [58, 59]. Therefore, the c and ee_s are

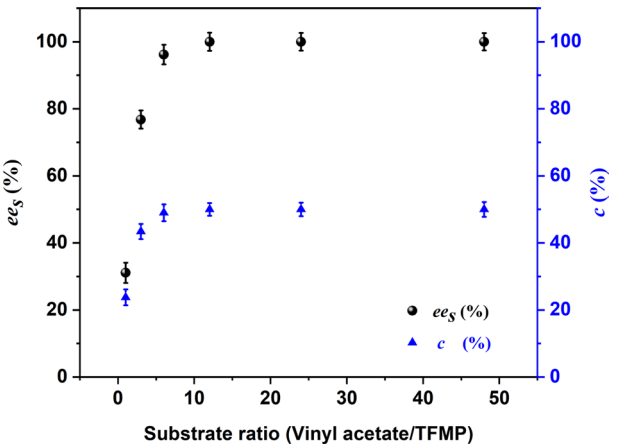

Fig. 4 Effect of substrate ratio on the resolution of TFMP enantiomers. Conditions: 5 mmol L^{-1} (*R,S*)-TFMP; 15 mg lipase PS; 2 mL isooctane; $T = 45$ °C; $t = 720$ min

rapidly increased. When a large amount of vinyl acetate is added, the available catalytic active sites are saturated and the further increase in the addition of vinyl can't improve the conversion.

Effect of time

Time is also an important factor for the lipase-catalyzed kinetic resolution. It is proposed to terminate the reaction at an appropriate time, where the fast-reaction enantiomer is just completely transformed, while the slow-reaction enantiomer has only the minimum amount of transformation. As can be seen from Fig. 5, ee_s and c increase gradually with the time first and then the growth becomes very slow after 80 min of reaction. When the reaction time was 110 min, ee_s and c reached the maximum and keep nearly unchanged with the further increase of time.

Kinetic study

Kinetic study was carried out through the method mentioned above. A constant concentration of vinyl acetate was set (much higher than TFMP) and the reaction was terminated at the initial stage of the reaction to evaluate the initial reaction rate, therefore, it can be approximately considered that the concentration of vinyl acetate remained constant during the reaction and the reaction was a single-substrate reaction. Fig. 6 shows the plots of V_0 versus $[(R)\text{-TFMP}]_0$, in which the experimental results (presented as scattered symbols) are compared graphically with the calculated values (presented as solid line). The experimental data was fitted to Michaelis–Menten equation through the non-linear least squares fitting method [60]. It is found that the calculated values agree well with the experimental results. The kinetic parameters K_m and V_{max} were estimated (Table 6) through the non-linear regression

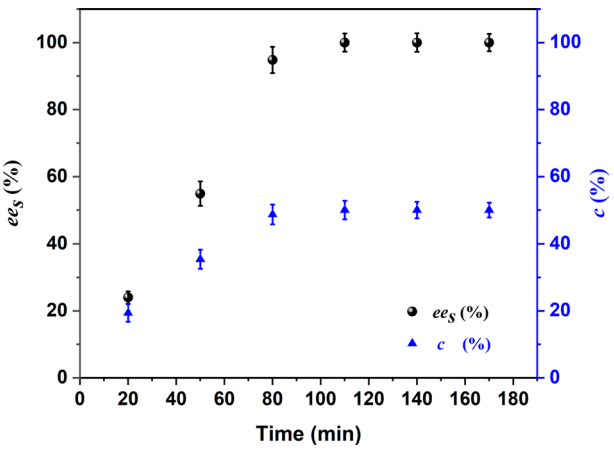

Fig. 5 Effect of time on the resolution of TFMP enantiomers. Conditions: 5 mmol L^{-1} (*R,S*)-TFMP; 60 mmol L^{-1} vinyl acetate; 15 mg lipase PS; 2 mL isooctane; $T = 45$ °C

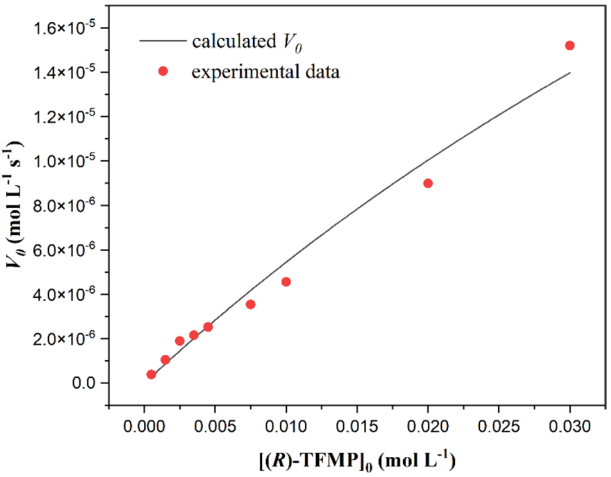

Fig. 6 The plot of V_0 versus [(*R*)-TFMP]$_0$. Solid line: calculated V_0; symbols: experimental data of V_0

Table 6 Apparent kinetic parameters

Lipases	K_m (mol L^{-1})	V_{max} (mol L^{-1} s^{-1})
Free lipase PS	0.11	6.41×10^{-5}

analysis with a R^2 value of 0.98. The parameters K_m and V_{max} are comparable with the results in the literature [61, 62].

Regression model and statistical analysis

The single-factor experiments show that four factors, namely temperature, substrate ratio, enzyme dosage and time, largely influence the resolution efficiency. However, it is hard to obtain the optimized conditions on the basis of the single-factor experiments. To further optimized the conditions, Box–Behnken experiment was designed with enantiomer excess and conversion as response values. Design-Expert software was used to conduct response surface regression analysis on the experimental data (Table 7), and the quadratic multinomial regression model of enantiomer excess and conversion and all investigated variables was obtained as follows:

$$ee_s\,(\%) = 100 + 13.06\mathrm{A} + 28.85\mathrm{B} + 13.7\mathrm{C} + 8.31\mathrm{D} - 0.35\mathrm{AB} - 13.35\mathrm{AC} - 9.87\mathrm{AD} + 12.1\mathrm{BC} - 0.15\mathrm{BD} - 5.7\mathrm{CD} - 4.49\mathrm{A}^2 - 38.03\mathrm{B}^2 - 12.55\mathrm{C}^2 - 3.07\mathrm{D}^2 \quad (7)$$

$$c(\%) = 50 + 2.75\mathrm{A} + 7.28\mathrm{B} + 1.94\mathrm{C} + 1.66\mathrm{D} - 2.85\mathrm{AB} - 3.52\mathrm{AC} - 2.97\mathrm{AD} + 0.45\mathrm{BC} - 0.5\mathrm{BD} - 1.5\mathrm{CD} + 0.95\mathrm{A}^2 - 8.32\mathrm{B}^2 - 1.51\mathrm{C}^2 + 0.19\mathrm{D}^2 \quad (8)$$

The ANOVA for the model is shown in Tables 8. It is found that F of ee_s regression model is 81.1 with $P < 0.0001$, F of c regression model is 46.74 with $P < 0.0001$,

Table 7 Box–Behnken Design Matrix of the independent variables with their corresponding response

Run	Temperature (°C)	Substrate ($\times 10^{-3}$ mmol)	Enzyme dosage (mg)	Reaction time (h)	c (%)	ee_s (%)
1	25	1	15	110	12.4	28.5
2	65	1	15	110	44.1	41.2
3	25	24	15	110	69.7	50.6
4	65	24	15	110	100.0	51.9
5	45	12.55	5	60	55.5	45.2
6	45	12.5	25	60	93.0	50.5
7	45	12.5	5	160	85.3	50.7
8	45	12.5	25	160	100.0	50.0
9	45	12.5	15	60	59.6	45.1
10	65	12.5	15	60	100.0	54.4
11	25	12.5	15	160	99.1	54.6
12	65	12.5	15	160	100.0	52.0
13	45	1	5	110	17.5	32.0
14	45	24	5	110	46.4	43.4
15	45	1	25	110	22.7	36.8
16	45	24	25	110	100.0	50.0
17	25	12.5	5	110	46.6	40.2
18	65	12.5	5	110	100.0	53.4
19	25	12.5	25	110	100.0	50.0
20	65	12.5	25	110	100.0	50.0
21	45	1	15	60	24.9	31.1
22	45	24	15	60	88.6	47.1
23	45	1	15	160	36.9	36.0
24	45	24	15	160	100.0	50.0
25	45	12.5	15	110	100.0	50.0
26	45	12.5	15	110	100.0	50.0
27	45	12.5	15	110	100.0	50.0
28	45	12.5	15	110	100.0	50.0
29	45	12.5	15	110	100.0	50.0

indicating that the significance of the two models is very high. At the same time, correlation coefficient R^2 of ee_s equation is evaluated as 0.9878, indicating that this model can be used to explain 98.78% of the change in response values, and correlation coefficient R^2 of c equation is evaluated as 0.9791, indicating that this model can be used to explain 97.91% of the change in response values, indicating that the two test models are in good fit and the model has high reliability.

In addition, the P-value can be used to check the significance of each coefficient. By comparing the P values of each factor. In this case, A, B, C, D, AC, AD, BC, CD, A^2, B^2, C^2 were significance for model ee_s. A, B, C, D, AC, AD, BC, B^2, C^2 were significant for model c, and the order of significance was $B > A = C > D$. The

Table 8 ANOVA for response surface reduced quadratic model

ee_s					c				
Source	Mean square	*F* value	*P*-value Prob > *F*		Source	Mean square	*F* value	*P*-value Prob > *F*	
Model	1909.8	81.1	< 0.0001	Significant	Model	102.94	46.74	< 0.0001	Significant
A	2026.24	86.89	< 0.0001	**	A	90.75	41.21	0.0002	**
B	9987.87	424.11	< 0.0001	**	B	636.56	289.04	< 0.0001	**
C	2252.28	95.64	< 0.0001	**	C	45.24		0.0002	**
D	828.34	35.17	< 0.0001	**	D	33	7.19	0.0179	*
AB	0.49	0.021	0.8874		AB	32.49	0.026	0.8746	
AC	712.89	30.27	< 0.0001	**	AC	49.7	5.02	0.0419	*
AD	390.06	16.56	0.0011	**	AD	35.4	6.3	0.0249	*
BC	585.64	24.87	0.0002	**	BC	0.81	7.24	0.0175	*
BD	0.09	3.82E-3	0.9516		BD	1	0.091	0.7676	
CD	129.96	5.52	0.034	*	CD	9	1.3	0.2734	
A^2	130.87	5.56	0.0335	*	A^2	5.85	0.98	0.3399	
B^2	9380.87	398.34	< 0.0001	**	B^2	449.55	95.42	< 0.0001	**
C^2	1022.32	43.41	< 0.0001	**	C^2	1117.4	11.27	0.0047	**
D^2	61	2.59	0.1298		D^2	58.38	0.59	0.4556	

*Represents significant influence (P < 0.05)

**Indicates extremely significant influence (P < 0.01)

Table 9 Predicted and observed values for the response variables based on optimal conditions

Response variable	Predicted value (%)	Experimental value (%)	Relative average deviation (%)
ee_s	100	99.6	0.2
c	50	49.5	1.1

Conditions: 5 mmol L^{-1} (*R,S*)-TFMP; 60 mmol L^{-1} vinyl acetate; 15 mg lipase PS; 2 mL isooctane; $T = 46$ °C; $t = 104$ min

following discusses the multi-factors interactions of the important factors based on the regression model.

Influence of substrate ratio and temperature

Fig. 7 shows the cross-impact of substrate ratio and temperature. From Fig. 7a, at a fixed substrate ratio, ee_s gradually increases with increasing temperature, however the increasing is not very significant. At lower substrate ratios (< 1:12), the effect of temperature on ee_s is smaller, e.g., when the substrate ratio is 1:1, ee_s changes from 12.4% to about 40% with increasing temperature. However, with the substrate ratio increases, the ee_s increases significantly and then decreases slowly. From Fig. 7b, the adjustment of substrate ratio can lead to an obvious change in c. It is observed that the enhanced ee_s and c can be achieved when the temperature is about 45 °C and substrate ratio is about 1:12.5.

Influence of time and substrate ratio

Fig. 8 shows the interaction of time and substrate ratio on the performance of lipase-catalyzed resolution at a fixed temperature of 45 °C. When the time increases from

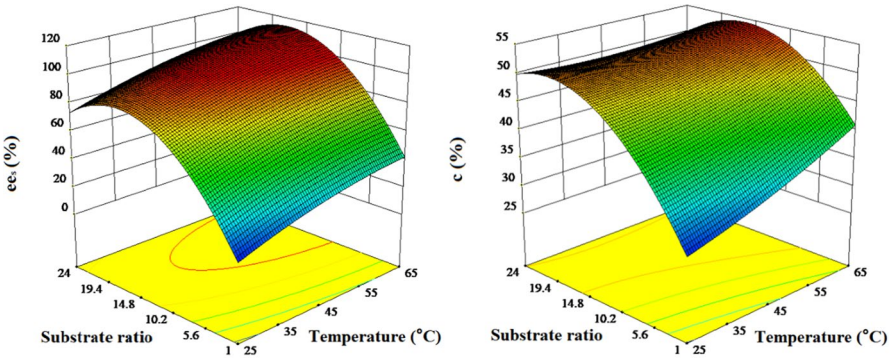

Fig. 7 Influence of substrate ratio and temperature on *ee*s (**a**) and *c* (**b**). Conditions: (*R,S*)-TFMP, 5 mmol L^{-1}; lipase PS, 15 mg; $t = 110$ min; isooctane, 2 mL

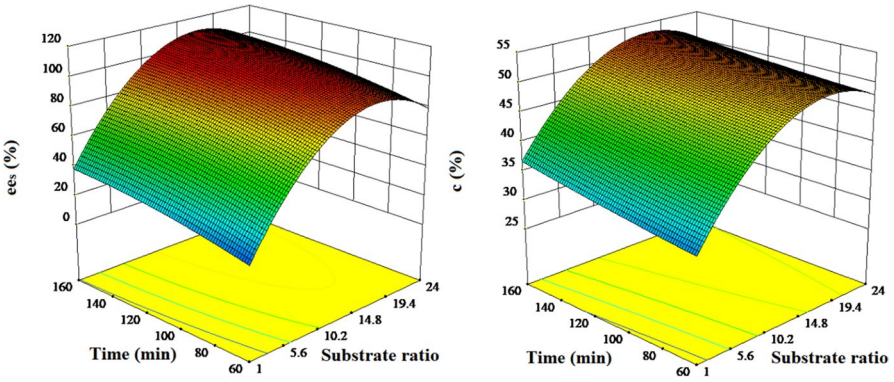

Fig. 8 Influence of time and substrate ratio on ee_s (**a**) and c (**b**). Conditions: (*R*,*S*)-TFMP, 5 mmol L^{-1}; lipase PS, 15 mg; $T = 45$ °C; isooctane, 2 mL

60 to 160 min, c and ee_s show a slight increase, showing an upward trend, while when the substrate ratio is increase from 1:1 to 1:24, c and ee_s increase significantly at first and then decrease slightly, the maximum value is reached around the substrate ratio of about 1:12.5. It shows that the increase of the substrate ratio has a strong effect on c and ee_s. The time of 110 min is proposed to achieve the optimized c and ee_s.

Influence of enzyme dosage and substrate ratio

As is shown in Fig. 9, with the increase of substrate ratio, ee_s and c show a trend of increase first and then decreases a little. The results are consistent with that observed in Fig. 9. On the other hand, ee_s and c are slightly enhanced by the increase of enzyme dosage. When the enzyme dosage is higher than 15 mg, the increase of c and ee_s with the enzyme dosage is slight. This indicates that continuing to increase the enzyme dosage has less effect on increasing c and ee_s values, so the amount of enzyme is kept at about 15 mg.

Influence of time and enzyme dosage

Fig. 10 shows the interaction of enzyme dosage and time on the resolution efficiency at a fixed temperature of 45 °C and substrate ratio of 12.5:1. It is observed that when the time is between 60 and 160 min and the enzyme dosage is between 5 and 25 mg, c and ee_s showed a trend of gradual increase with the increase of time and enzyme dosage. The optimal c and ee_s can be achieved in region where time is higher than 110 min and the enzyme dosage is higher than 15 mg.

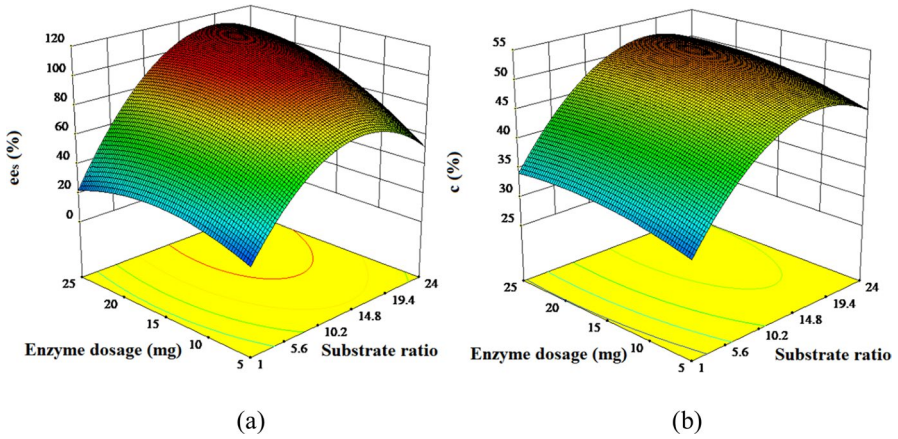

Fig. 9 Influence of enzyme dosage and substrate ratio on ee_s (**a**) and c (**b**). Conditions: (*R,S*)-TFMP, 5 mmol L^{-1}; $T = 45$ °C; $t = 110$ min; isooctane, 2 mL

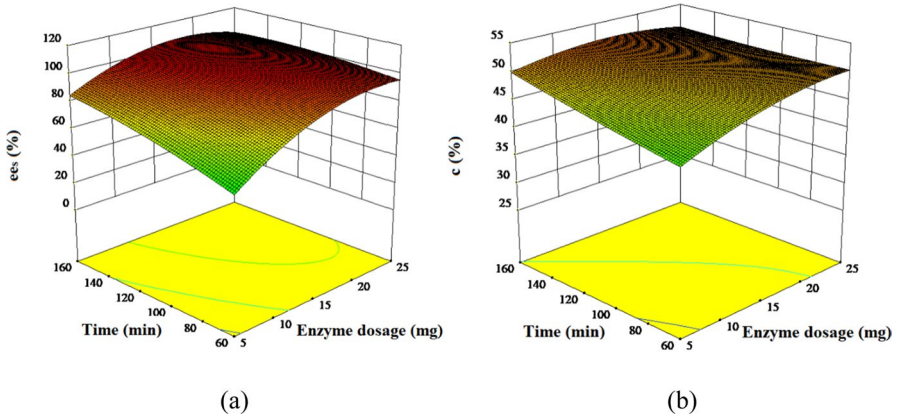

Fig. 10 Influence of time and enzyme dosage on ee_s (**a**) and c (**b**). Conditions: (*R,S*)-TFMP, 5 mmol L^{-1}; vinyl acetate, 62.5 mmol L^{-1}; $T = 45$ °C; isooctane, 2 mL

Application and validation of the model

Based on the regression model, the optimal conditions for kinetic resolution of TFMP enantiomers by lipase-catalyzed transesterification are obtained as follows: temperature of 46 °C, substrate ratio of 1:12, enzyme dosage of 15 mg, time of 104 min. Under these conditions, the ee_s higher than 99.0% and c of about 50.0% are expected to be obtained. The chromatograms of TFMP are shown in Fig. 11a, b respectively. As shown in Table 9, three parallel verification experiments were carried out under the optimal conditions, and the measured ee_s higher than 99.5% and c of 50.3% are achieved (reported with the average value), which proves the validity of the model.

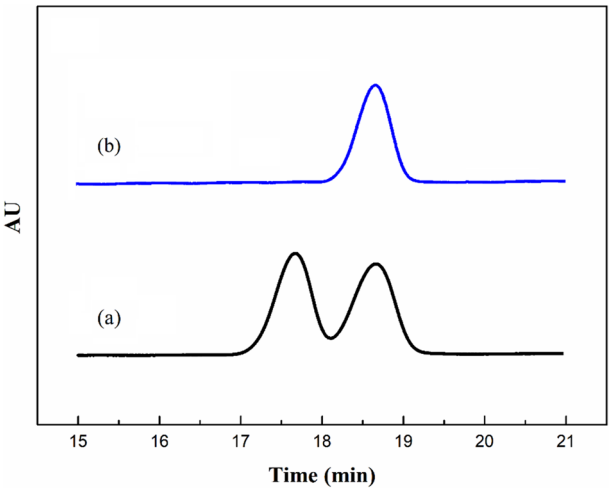

Fig. 11 Chromatograms of (*R, S*)-TFMP and remaining substrate by HPLC. **a** Sample of TFMP racemate, **b** Sample of the remaining substrate

Although good results for the kinetic resolution of TFMP enantiomers with lipase PS are achieved, the powder of lipase PS is hardly able to be reused, because the powder is only partially recovered from the reaction system and the expressed activity of the recycled powder is considerably reduced. In order to further improve the industrial application ability of lipase PS, more attention should be paid to the research of enzyme immobilization.

Conclusion

Kinetic resolution of TFMP enantiomers through lipase-catalyzed transesterification in an organic solvent was performed. Lipase PS was selected as the biocatalyst and isooctane was selected as the organic solvent. Single-factor experiments were performed to identify the important factors that influence the resolution efficiency. The results showed that ee_s and c mainly depended on substrate ratio, temperature, enzyme dosage and time. the RSM with Box-Behnken experimental designation was employed for further investigation and optimization of the reaction conditions. Results showed that the regression models for ee_s and c are significant and the optimized conditions were obtained. Under the optimized conditions including temperature of 46 °C, substrate ratio of 1:12, enzyme dosage of 15 mg and time of 104 min, TFMP enantiomers were efficiently resolved with ee_s higher than 99.0% and c of 50.3%. The resolution system has good industrial application potential.

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