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Optimization of lipase-catalyzed enantioselective esterification of (±)-menthol in ionic liquid

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Abstract

Response surface methodology was successfully applied to optimize lipase-catalyzed enantioselective esterification of (±)-menthol. The effects of various reaction conditions, including reaction time, temperature, enzyme loading, substrate molar ratio and water activity, were investigated. A Central Composite Rotatable Design was employed to search for the optimal conversion of (±)-menthol and enantiomeric excess. A quadratic polynomial regression model was used to analyze the experimental data at a 95% confidence level ($p < 0.05$). The analysis confirmed that reaction temperature, enzyme loading and reaction time were the significant factors affecting the conversion of (±)-menthol. Moreover, reaction temperature, enzyme loading, substrate molar ratio and reaction time were found to affect the enantiomeric excess significantly. The coefficient of determination of these two models was found to be 0.980 and 0.967, respectively. Two sets of optimum reaction conditions were established and the verified experimental trials were performed for validating the optimum points. Under the optimum conditions, the conversion of (±)-menthol and the enantiomeric ratio exceeded 53% and 40%, respectively.
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Keywords: Menthol; Enantioselective esterification; Response surface methodology; Conversion; Enantiomeric excess; Optimization

1. Introduction

(−)-Menthol, a menthol isomer, is a component of peppermint oil. Because of its cooling and refreshing effect, (−)-menthol is an important fragrance and flavor compound and is used widely in cosmetics, toothpaste, chewing gums, cigarettes, sweets and medicines (Wang, Nag, Lee, & Shaw, 2002). In general, it is produced by optimal resolution of (±)-menthol which is synthesized chemically in the industry (Shimada et al., 1999). Enzymatic esterification is a highly selective method for the resolution of (±)-menthol. Recently, enzymatic resolution of (±)-menthol in organic solvent has been investigated by some researchers (Athawale, Manjrekar, & Athawale, 2002; Wang et al., 2002; Yuan, Bai, & Sun, 2006). Among several lipases investigated, lipase from *Candida rugosa* were found to be highly selective for the esterification of (−)-menthol (Yuan et al., 2006).

In recent years, room-temperature ionic liquids (compounds that consist only of ions and are liquid at room temperature) have increasingly attracted attention as the green, high-tech reaction media of the future (Kim, Choi, Lee, & Ahn, 2003; Nara, Mohile, Harjani, Naik, & Salunkhe, 2004). Their versatility as solvents for such diverse applications is due to the favorable attributes of an 'ideal solvent' that they possess (Cull, Holbrey, Vargas-Mora, Seddon, & Lye, 2000; Guo & Xu, 2006). Ionic liquids have only recently become attractive as a new type of alternative media for enzymatic reactions since this medium is known to have positive influences not only on the enzyme-stability and activity, but also on enantioselectivity (Kim, Song, Choi, & Kim, 2001; Schofer, Kaftzik, Wasserscheid, & Kragl, 2001). We investigated previously the enantiomeric resolution of racemic menthol in a hydrophobic ionic liquid, [bmim] PF_6, and the results showed that lipase-catalyzed esterification of (±)-menthol proceeded efficiently in ionic

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liquids with superior enantioselectivity to those performed in conventional organic solvents (Yuan et al., 2006).

Statistical optimization method overcomes the limitations of classic empirical methods and proves to be a powerful tool for the optimization of the target value (Garrido-Vidal, Pizarro, & Gonzalez-Saiz, 2003; Zanto, Al-Muhtaseb, & Ritter, 2002). Response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for designing experiments, building models and analyzing the effects of the several independent variables (factors) (Garrido-Vidal et al., 2003; Zhang, Bai, Dong, & Sun, 2007). The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple factors and their interactions. The study of the individual and interactive effects of these factors will be helpful in efforts to find the target value. Hence, RSM provides an effective tool for investigating the aspects affecting desired response if there are many factors and interactions in the experiment. In order to determine a suitable polynomial equation for describing the response surface, RSM can be employed to optimize the process.

The present work focuses on the parameters that affect lipase from *Candida rugosa* to catalyze the enantioselective esterification of (−)-menthol for separating (±)-menthol using propionic anhydride as the acyl donor in an ionic liquid. Our purpose is to better understand the relationships between the factors (reaction time, temperature, enzyme loading, substrate molar ratio, and water activity) and the response (enantiomeric excess (*e.e.*%), and enantiomeric ratio (*E*)); also to determine the optimal conditions for enantiomeric resolution of (±)-menthol using central composite rotatable design (CCRD) and response surface methodology (RSM).

2. Experimental

2.1. Materials

(±)-Menthol, (−)-menthol, propionic anhydride, (−)-menthyl propionate and *Candida rugosa* lipase (CRL, Type VII) were purchased from Sigma (St. Louis, MO, USA). Methanol, hexane and dichloromethane for gas chromatography were of HPLC grade from Fisher Scientific (Fair Lawn, NJ, USA). All other regents were of analytical grade and obtained from local sources. The organic solvents were anhydrated by molecular sieves of 3 Å (Dalian Institute of Chemical Physics, Dalian, China) before use.

2.2. Preparation and characterization of ionic liquids

The ionic liquid 1-butyl-3-methyl imidazolium hexafluorophosphate ([bmim] PF_6) was prepared according to the procedure described by Huddleston (Huddleston & Rogers, 1998). The [bmim] PF_6 was purified according to the procedure described by Park and Kazlauskas (Park & Kazlauskas, 2001) and the purity was determined by elemental analysis.

2.3. Esterification of menthol

All enzymatic reactions were carried out in a temperature-controlled incubator shaker. In a typical experiment, 1.0 mmol of (±)-menthol and 399 IU of CRL were added to 3 ml of the ionic liquid in a 10 ml screw-capped vial. The reaction was started by adding 1.0 mmol of propionic anhydride and run by shaking at 200 rpm at designated temperature. Samples (300 μl) of the biotransformation were withdrawn at different times, then suspended in 1 ml of hexane/10% $NaHCO_3$ (1:1, v/v). The multiphasic mixture was rigorously shaken to extract all substrates and product to the hexane phase and remove acid to the aqueous phase. Then, 100 μl hexane extract were diluted with 500 μl hexane, and 1 μl of the diluted solution was analyzed by gas chromatography (GC).

Control experiments were performed in the absence of CRL. As a result, no chemical acyl transfer reaction was detected.

2.4. GC analysis

The GC analysis was performed with an Agilent 6890N GC (Agilent Technologies, DE, USA) equipped with a splitless/split injector, a flame-ionization detector, and a CYCLOSIL-B capillary column (0.25 μm film thickness, 30 m length, 0.25 mm I.D.). The injector and detector were set at 200 and 250 °C, respectively, and the flow rate of the carrier gas N_2 was 2 ml min^{-1}. The oven temperature was kept at 90 °C for 10 min, programmed to increase from 90 to 150 °C at 2 °C/min, then increased to 165 °C at 5 °C/min, and finally kept at 165 °C for 5 min. Chromatographic data were acquired and analyzed using the Agilent Chemical Station. The retention time were found to be 30.5 and 31.5 min for (−)-menthol and (+)-menthol propionate, respectively, and 26.3 min for (±)-menthol.

2.5. Calculation of enantioselectivity

The enantiomers of the (±)-menthol and of the product (±)-menthyl propionate were baseline separated in the GC analysis. The conversion in percentage was calculated from the following equation:

$$C = \frac{P_- + P_+}{P_- + P_+ + S} \times 100\% \quad (1)$$

The enantioselectivity for each reaction was expressed by enantiomeric excess ($e.e._P$%) and enantiomeric ratio (*E*-value).

$$e.e._P = \frac{P_- - P_+}{P_- + P_+} \times 100\% \quad (2)$$

$$E = \frac{\ln(1 - c(1 + e.e._P))}{\ln(1 - c(1 - e.e._P))} \quad (3)$$

where *S*, P_- and P_+ stand for menthol and the products of (−) and (+)-menthyl propionate, respectively.

2.6. Water activity (a_w) control and measurement

The prepared [bmim] PF_6 was first dried by vacuum of 1 mm Hg for 10 h. Then a series of mixtures with substrates in specific water content were prepared by adding a certain amount of water. The resulting samples were pre-equilibrated for 24 h with magnetic stirring in a sealed vial before being subjected to a_w measurement. The water activity (a_w) of the ionic liquid with specific water content was measured with Hygrolab Humidity Detector (Rotronic, Swiss) before being applied to the reaction.

2.7. Experimental design

Response surface methodology (RSM) was employed to analyze the operating conditions of menthol acylation to obtain a high percent conversion and high enantiomeric excess. The experimental design was carried out by five chosen independent process variables at five levels (Table 1). The studied factors were: reaction time (t, (h)), reaction temperature (T, (°C)), enzyme loading (IU/ml), substrate molar ratio (Menthol: Propionic anhydride, MR) and water activity (a_w). For each factor, the experimental range and the central point were shown in Table 1.

The software of Design-Expert 6.0 (Stat-Ease, USA) was used for designing and analyzing the experimental data. The coded values of these factors were obtained according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \tag{4}$$

where x_i is the coded value of the factor, X_i is the real value of the factor, X_0 is the real value of the factor at the center point, and ΔX_i is the step change value of the factor.

The independent variables (factors) and their levels, real values as well as coded values are presented in Table 1. The enantiomeric excess ($e.e._P$%) and the percent conversion of menthol (c%) were the responses of the experimental design.

The model equation was used to predict the optimum value and subsequently to elucidate the interaction between the factors. The quadratic equation model for predicting the optimal point was expressed according to Eq. (5):

$$Y = \beta_0 + \sum_{i=1}^{5} \beta_i x_i + \sum_{i=1}^{5} \beta_{ii} x_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} \beta_{ij} x_i x_j \tag{5}$$

where β_0, β_i, β_{ii}, and β_{ij} are regression coefficients (β_0 is constant term, β_i is linear effect term, β_{ii} is squared effect term, and β_{ij} is interaction effect term), and Y is the predicted response value.

Table 1
Coded levels for independent factors used in the experimental design

Factors	Symbol	Coded levels				
		−2	−1	0	1	2
Reaction time (h)	x_1	4	6	8	10	12
Reaction temperature (°C)	x_2	20	25	30	35	40
Enzyme loading (IU/ml)	x_3	67	100	133	166	200
Substrate molar ratio (MR)	x_4	0.3	0.65	1	1.35	1.7
Water activity (a_w)	x_5	0.03	0.08	0.13	0.18	0.23

3. Results and discussion

3.1. Effect of reaction time

Fig. 1 shows the time course for the enantioselective esterification of (±)-menthol by CRL at 30 °C. The conversion of menthol increased to 53% after 8 h; therefore, the range of reaction time from 4 to 12 h was chosen in this study. The selection of reaction time range had to be extremely precise in the study of CCRD, otherwise, the optimal condition of synthesis could not be found within the experimental region through the analyses of statistics and contour plots. Also, as shown in Fig. 1, the $e.e._P$ exceeded 90% at initial stage of the reaction, and then followed a slight decline. The reason was that (−)-menthol was esterified preferentially initially, and with the proceeding of the enantioselective esterification of (±)-menthol, the reaction probability of (+)-menthol increased due to the increasing consumption of (−)-menthol.

3.2. RSM experiments and fitting the models

A Central Composite Rotatable Design (CCRD) was employed to design the experiments. According to statistical theory, a CCRD design of five factors consists of 30 experiments, including 15 factorial points (cubic point) and 11 axial points (star point) as well as four replicates

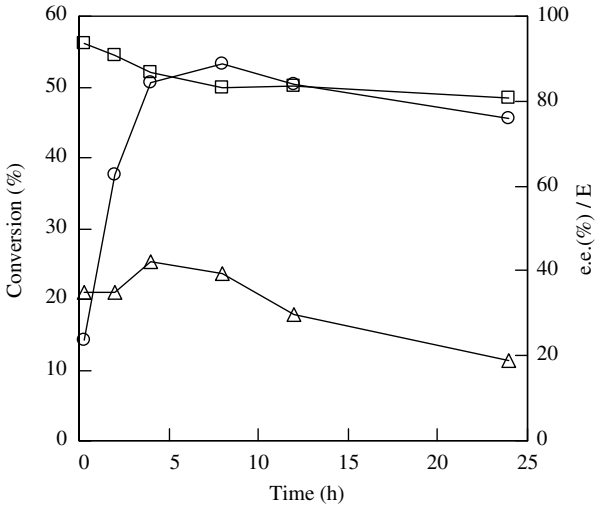

Fig. 1. Time course of the enantioselective esterification of menthol in [bmim] PF_6. (○, percent conversion of (±)-menthol; □, $e.e._P$%; Δ, E-value) (Reaction conditions: ionic liquid 3 ml, temperature 30 °C, $a_w = 0.13$, Menthol/Propionic anhydride mole ratio 1:1, 133 IU/ml CRL).

at the center point. Four replications (treatments 27–30) at the centre of the design were used to estimate the pure error. The results at each point based on experimental design are shown in Table 2, which also gives the experimental data of the response value, $e.e._P\%$ and $c\%$. The coded values of each factor in brackets correspond to the real value of the factor levels. For each factor, a conventional level was set at zero as a coded level. The runs were randomized for statistical reasons.

3.2.1. The conversion of menthol ($c\%$)

The effects of factors as well as their interactions on the $c\%$ could be discussed from the Pareto chart illustrated by Fig. 2. The length of each bar was proportional to the absolute value of its associated regression coefficient or estimated effect (Brand et al., 2001). The order in which the bars were displayed corresponded to the order of the size of the effect. The chart included a vertical line that corresponded to the 95% limit indicating statistical significance. A factor was, therefore, significant if its corresponding bar crossed this vertical line (Brand et al., 2001). As indicated in Fig. 2, several different conclusions could be obtained: (1) The conversion of menthol was greatly affected by reaction temperature (x_2), enzyme loading (x_3), reaction time (x_1), and a quadratic terms of x_5^2, x_2^2, x_3^2, x_4^2, x_1^2. Likewise, significant interactions were also found between enzyme loading and substrate molar ratio (x_3x_4) ($p < 0.05$); (2) The second-order effects of a_w

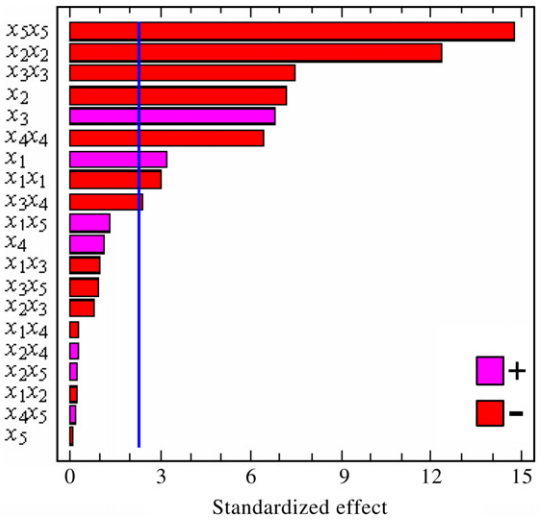

Fig. 2. Pareto chart of standardized effects for the model of percent conversion. Positive effects are in pink and negative effects are in red. The line indicates the confidence level of 95%, and factors with standardized effect values to the right of this line are statistically significant.

Table 2
Experimental design and results of the 1/2 CCRD design

Trial	Variable level					Response value	
	t (h)	T (°C)	Enzyme (mg/ml)	MR	a_w	$c\%$	$e.e._P\%$
1	6 (−1)	25 (−1)	100 (−1)	0.65 (−1)	0.18 (1)	23.25	85.98
2	10 (1)	25 (−1)	100 (−1)	0.65 (−1)	0.08 (−1)	29.04	84.82
3	6 (−1)	35 (1)	100 (−1)	0.65 (−1)	0.08 (−1)	17.40	73.77
4	10 (1)	35 (1)	100 (−1)	0.65 (−1)	0.18 (1)	23.62	68.23
5	6 (−1)	25 (−1)	166 (1)	0.65 (−1)	0.08 (−1)	38.82	84.80
6	10 (1)	25 (−1)	166 (1)	0.65 (−1)	0.18 (1)	39.94	86.52
7	6 (−1)	35 (1)	166 (1)	0.65 (−1)	0.18 (1)	26.14	82.00
8	10 (1)	35 (1)	166 (1)	0.65 (−1)	0.08 (−1)	30.42	79.84
9	6 (−1)	25 (−1)	100 (−1)	1.35 (1)	0.08 (−1)	29.84	84.85
10	10 (1)	25 (−1)	100 (−1)	1.35 (1)	0.18 (1)	35.75	83.33
11	6 (−1)	35 (1)	100 (−1)	1.35 (1)	0.18 (1)	22.84	69.98
12	10 (1)	35 (1)	100 (−1)	1.35 (1)	0.08 (−1)	26.00	66.04
13	6 (−1)	25 (−1)	166 (1)	1.35 (1)	0.18 (1)	34.15	85.95
14	10 (1)	25 (−1)	166 (1)	1.35 (1)	0.08 (−1)	38.42	82.70
15	6 (−1)	35 (1)	166 (1)	1.35 (1)	0.08 (−1)	28.95	79.05
16	10 (1)	35 (1)	166 (1)	1.35 (1)	0.18 (1)	29.88	75.42
17	4 (−2)	30 (0)	133 (0)	1.00 (0)	0.13 (0)	45.70	86.72
18	12 (2)	30 (0)	133 (0)	1.00 (0)	0.13 (0)	50.33	83.59
19	8 (0)	20 (−2)	133 (0)	1.00 (0)	0.13 (0)	36.21	80.56
20	8 (0)	40 (2)	133 (0)	1.00 (0)	0.13 (0)	22.38	63.52
21	8 (0)	30 (0)	67 (−2)	1.00 (0)	0.13 (0)	32.06	80.39
22	8 (0)	30 (0)	200 (2)	1.00 (0)	0.13 (0)	46.05	85.74
23	8 (0)	30 (0)	133 (0)	0.30 (−2)	0.13 (0)	41.89	86.91
24	8 (0)	30 (0)	133 (0)	1.70 (2)	0.13 (0)	40.41	79.15
25	8 (0)	30 (0)	133 (0)	1.00 (0)	0.03 (−2)	23.78	74.04
26	8 (0)	30 (0)	133 (0)	1.00 (0)	0.23 (2)	25.05	81.73
27	8 (0)	30 (0)	133 (0)	1.00 (0)	0.13 (0)	53.24	83.16
28	8 (0)	30 (0)	133 (0)	1.00 (0)	0.13 (0)	53.02	81.16
29	8 (0)	30 (0)	133 (0)	1.00 (0)	0.13 (0)	51.32	80.92
30	8 (0)	30 (0)	133 (0)	1.00 (0)	0.13 (0)	49.76	80.69

(x_5^2) and reaction temperature (x_2^2) were more significant than their respective first-order effects. (3) The regression coefficient of reaction temperature was negative, which suggested that too high temperature would not benefit the conversion of menthol. Similarly, the effects of the terms would be positively correlated if the coefficients were positive. According to the statistical method, the data were fitted to a response surface model to effectively evaluate the true relationship between the *e.e.*$_P$ and the factors. A quadratic regression model was obtained by using coded values from the estimation of data:

$$Y_1 = 52.60 + 1.71x_1 - 3.82x_2 + 3.65x_3 - 1.54x_1^2 - 6.22x_2^2 - 3.73x_3^2 - 3.25x_4^2 - 7.44x_5^2 - 1.56x_3x_4 \quad (6)$$

where x_i is the coded value of each factor.

3.2.2. Enantiomeric excess

Similar to Fig. 2, Fig. 3 denotes the effects of factors as well as their interactions on enantiomeric excess. Comparing Fig. 3 with Fig. 2, we can draw several conclusions: (1) reaction temperature was the most significant factor affecting the enantiomeric excess; (2) substrate molar ratio produced a significant effect on the enantiomeric excess, although it was not important for the conversion of menthol; (3) also, significant interaction was found between temperature and enzyme loading. As aforementioned, the data were fitted to a response surface model to effectively evaluate the true relationship between enantiomeric excess and the factors. A quadratic regression model was obtained by using coded values from the estimation of data:

$$Y_1 = 82.59 - 1.07x_1 - 4.95x_2 + 2.08x_3 - 1.42x_4 - 2.66x_2^2 - 1.12x_5^2 + 2.33x_2x_3 \quad (7)$$

where x_i is the coded value of each factor.

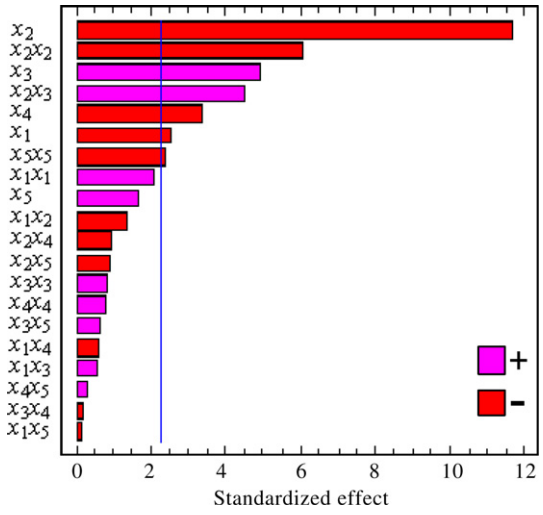

Fig. 3. Pareto chart of standardized effects for the model of enantiomeric excess. Positive effects are in pink and negative effects are in red. The line indicates the confidence level of 95%, and factors with standardized effect values to the right of this line are statistically significant.

3.3. Analysis of variance (ANOVA) and adequacy test of the models

For the model fitted, software generated model coefficients, *F*-values and *p*-values (*Prob* > *F*, which indicates the insignificant probabilities) and hence one could justify the significance of each experimental variable. The corresponding variable would be more significant if the absolute *F*-value became larger and the *p*-value became smaller (Amin & Anggoro, 2004). As can be seen from Tables 3 and 4, the regression quadratic models were both highly significant ($p < 0.0001$) and the lack of fit was insignificant ($p > 0.05$), which indicated that the two models were adequate to explain most of the variability for the *c*% and the *e.e.*$_P$, respectively.

To evaluate the optimization technique, the observed and predicted values of the *c*% were compared and the results are presented in Fig. 4. As can be seen, the predicted values of the response from the model accorded well with

Table 3
Analysis of variance (ANOVA) for the quadratic model for the *c*%

Source	Sum of square	DF	Mean square	*F*-value	*Prob* > *F*
Model	3104.22	20	155.21	22.54	<0.0001
Residual	61.98	9	6.89		
Lack of fit	54.03	6	9.01	3.40	0.1714
Pure Error	7.95	3	2.65		
Total	3166.20	29			

R^2 (coefficient of determination) of the model is 0.9804.

Table 4
Analysis of variance (ANOVA) for the quadratic model for the *e.e.*$_P$

Source	Sum of square	*DF*	Mean square	*F*-value	*Prob* > *F*
Model	1124.05	20	56.20	12.99	0.0002
Residual	38.94	9	4.33		
Lack of fit	35.08	6	5.85	4.54	0.1210
Pure Error	3.86	3	1.29		
Total	1162.99	29			

R^2 (coefficient of determination) of the model is 0.9665.

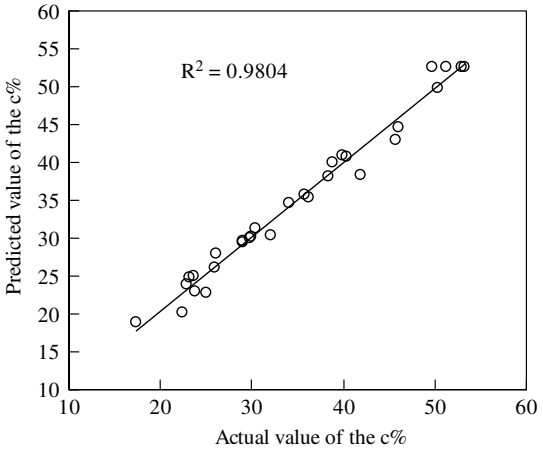

Fig. 4. Comparison between the predicted and the observed conversion of menthol.

the observed values. Consequently, this model could be used to navigate the design space.

3.4. Analysis of response surfaces

3.4.1. Mutual effect of factors on the conversion of menthol

The 3D-plots were drawn to illustrate the main and interactive effects of the independent variables on the conversion of menthol. The response surfaces based on these factors are shown in Fig. 5. Fig. 5a represents the 3D-plot of the effect of reaction time and temperature on the reaction. From the analysis of the response surface plots, reaction temperature exhibited a more significant influence on the response surface in comparison to reaction time. At initial time, the conversion of menthol increased as the temperature was enhanced, which reflected a general effect of ascending temperature on the reaction rate. Subsequently, the conversion of menthol emerged a peak with a maximum value around 28 °C and then declined, possibly because of the thermal denaturation of lipase (Zhang, Guo, Dong, & Sun, 2007).

Fig. 5b depicts the enzyme loading and substrate molar ratio effect on the response. As can be seen, enhancing enzyme loading could bring about high conversion of menthol, but excess amount of enzyme would influence the mass transfer of the reaction and led to the decline of the conversion of menthol. On the other hand, the increase of propionic anhydride amount, i.e. the decrease of substrate molar ratio, could increase the conversion of menthol. Excess acyl donor would lead to conversion decrease, which was probably caused by the substrate inhibition. In addition, significant interactions were found between enzyme loading and substrate molar ratio. In Fig. 5b, the response surface of menthol conversion shows a net peak of 53.49% at 156.41 IU/ml of enzyme loading and 1:1 of substrate molar ratio.

The effect of varying water activity on the reaction is shown in Fig. 5c. As described elsewhere (Yang, Wang, & Kuo, 2004), the effect of water content of the reaction system could be due to several reasons. On the one hand, water activity is one of the most important factors to influence the lipase activity and all kinds of lipase have an optimum a_w (Zhang, Bai, & Sun, 2007); on the other hand, the less the water content is, the greater the viscosity of ionic liquid [bmim] PF_6 is, and which would result in the great resistance of mass transfer. In the light of Fig. 5c, the

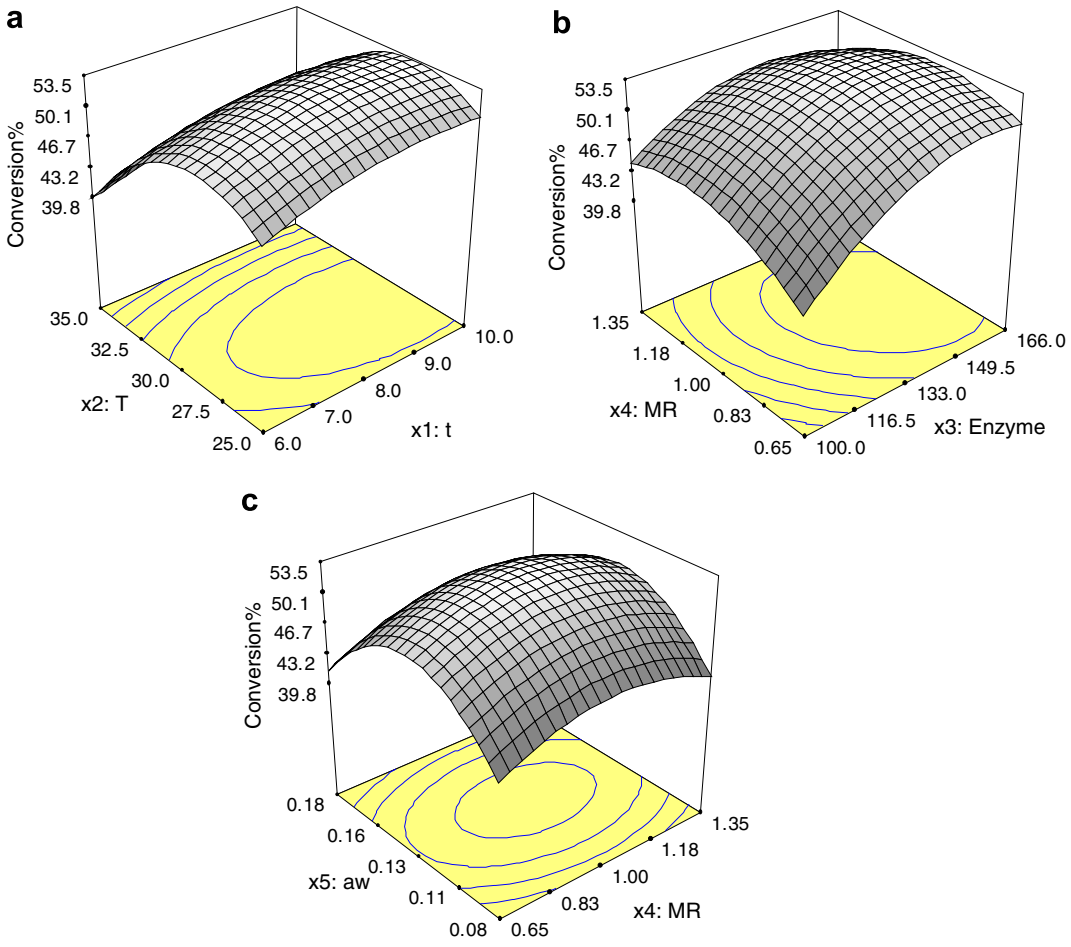

Fig. 5. 3D-plot between any two parameters for the conversion of menthol (Conditions: (a) Enzyme loading 133 IU/ml, MR = 1.00, $a_w = 0.13$; (b) $t = 8$ h, $T = 30$ °C, $a_w = 0.13$; (c) $t = 8$ h, $T = 30$ °C, Enzyme loading 133 IU/ml).

response surface shows a peak with 53.49% at optimum a_w 0.13.

Overall, reaction time, temperature and enzyme loading were the most important variables for the conversion of enantioselective esterification of (±)-menthol.

3.4.2. Mutual effect of factors on the enantiomeric excess

The effects of these five factors as well as their interactive effects on the enantiomeric excess can be seen from Fig. 6. Fig. 6a denotes the two-dimensional contour plots of the effect of reaction time and temperature. As indicated, reaction temperature performed a very significant influence on the enantiomeric excess, and the response was expected to exhibit a monotonic increase with decrease of temperature. That was to say, low temperature was more favorable for improving stereospecificity. One possible explanation was that low temperature could increase the "rigidity" of the lipase, which enhanced the enantioselective recognition capability of the stereospecificity "pocket" (Nakamura, Kinoshita, & Ohno, 1995; Uppenberg et al., 1995); while high temperature increased the "flexibility" of the lipase and therefore brought down the recognition capability, which was similar to the previous report (Phillips, 1996). In Fig. 6a, the enantiomeric excess shows a decreasing trend along with the reaction time course, which could be attributed to the fact that, with the proceeding of the reaction, the increasing consumption of (−)-menthol resulted in the incremental reaction probability of (+)-menthol.

The effect of enzyme loading on the enantiomeric excess is shown in Fig. 6b. As depicted, keeping other experimental conditions constant, the enantiomeric excess would slightly increase with enzyme loading. A reaction with enzyme concentrations of 155–165 IU/ml and reaction time of 9–10 h led to over 83% enantiomeric excess. Substrate molar ratio and a_w were investigated in the range of 0.65–1.35 and 0.08–0.18, respectively, and the effect of substrate molar ratio, a_w, and their mutual interaction on enantiomeric excess are shown in Fig. 6c. As can be seen, the effect of a_w on the response was not very significant, which is in agreement with Fig. 3. With the increase of substrate molar ratio, there was a slight decrease in the response value. As mentioned above, by using response surface methodology, the effect of these factors on enantioselective esterification of menthol could be studied.

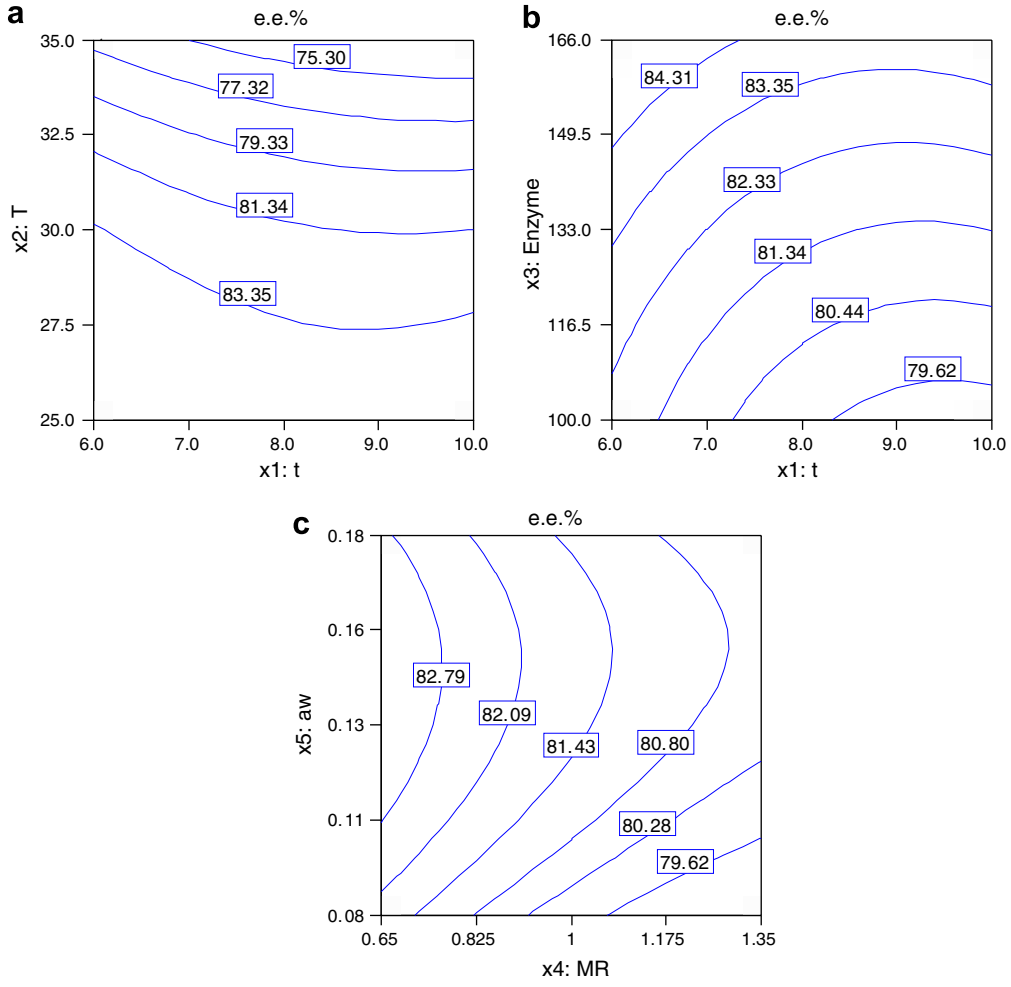

Fig. 6. Contour plots between any two parameters for the production enantiomeric excess of esterification of menthol (Conditions: (a) Enzyme loading 133 IU/ml, MR = 1.00, a_w = 0.13; (b) T = 30 °C, MR = 1.00, a_w = 0.13; (c) t = 8 h, T = 30 °C, Enzyme loading 133 IU/ml).

Table 5
Optimum conditions found by the model and verification of the model

Run	1 #	2 #
Reaction time (h)	9.53	10
Reaction temperature (°C)	27.64	27.52
Enzyme loading (IU/ml)	156.41	161.97
Molar ratio (MR)	1	0.91
Water activity (a_w)	0.13	0.14
Predicted value *c%e.e.%*	53.82	53.84
	84.79	84.78
Experimental value *c%e.e.%*	53.77	52.69
	84.04	84.35

3.5. Attaining optimum conditions and model verification

As we know, it is of general interest for developing industrial processes for the enantioselective esterification of (±)-menthol useful for food additives and cosmetic formulations as well as medicine industry. Based on the discussion above, it was possible to obtain a high degree of conversion and high enantiomeric ratio through searching for the optimum point. Hence, two sets of predicted reaction conditions are given by the model (Table 5).

To validate the predicted results, experiments using the improved formula were performed, and the observed values were shown in Table 5. Based on the solution given by the design, two runs of experiments were established at the fixed conditions. The experimental values were found to be reasonably close to the predicted ones, which confirmed the validity and adequacy of the predicted models. In addition, under these two sets of conditions, the enantiomeric ratios (E) have also been calculated by Eq. (3) and the E-values were 50.87 and 41.28, respectively, which were much higher than the previous report (Yuan et al., 2006).

4. Conclusions

Candida rugosa lipase was used as a biocatalyst to perform enantioselective esterification of (±)-menthol in hydrophobic ionic liquid [bmim] PF_6. Response surface methodology was successfully applied to determine the operation conditions for optimizing the conversion of menthol and enantiomeric ratio. The results showed a significantly good fit to this model, and the response evaluated from the quadratic model showed a good agreement with the observed ones. The F-test and p-value indicated that reaction temperature, enzyme loading and reaction time were the significant factors affecting the conversion of menthol. Moreover, reaction temperature, enzyme loading, substrate molar ratio and reaction time were found to affect the enantiomeric excess significantly. Two sets of optimum operation conditions were established. Furthermore, under optimized conditions the experimental values agreed well with the values predicted. By the optimum model, the conversion of menthol and the E-value could exceed 53% and 40, respectively. The experimental conditions allowed a fast, quantitative and maximum enantiomeric resolution of (±)-menthol.

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