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Full Length Research Paper

Optimization of enantioselective production of chiral epichlorohydrin catalyzed by a novel epoxide hydrolase from domestic duck liver by response surface methodology

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Enantiopure epichlorohydrin is a valuable epoxide intermediate for preparing optically active pharmaceuticals. In the present study, a novel epoxide hydrolase prepared from domestic duck liver was used as biocatalyst for producing (S)-epichlorohydrin which preparation process was optimized by response surface methodology. Response surface methodology was performed to evaluate the effects of reaction temperature, pH and reaction time on production of (S)-epichlorohydrin by the novel epoxide hydrolase. (S)-epichlorohydrin production was optimized by Box-Behnken. Three reaction parameters were optimized as follows: pH value 7.10, reaction temperature 32.44°C and reaction time 11.06 h. The adequately high R^2 value 0.9599 and F score 13.29 indicated the statistical significance of the model. The enantioselective excess of (S)-epichlorohydrin after optimization was 86.14% while the predicted value was 85.55%. In conclusion, enantioselective hydrolysis conditions optimization to enhance optical purity of (S)-epichlorohydrin could be easily and effectively done by response surface methodology; the developed production process indicated the novel epoxide hydrolase from domestic duck liver was high efficient biocatalyst for preparing enantiopure epichlorohydrin.

Key words: Chiral epichlorohydrin, enantioselective production, epoxide hydrolase, domestic duck liver, response surface methodology.

INTRODUCTION

The conventional “one-factor-at-a-time” approach of improving reaction conditions is the most frequently utilized operation in varieties of research fields to obtain maximum yield of product. However, this method is time consuming and frequently, disregards the complex interactions among various physicochemical parameters. In order to overcome these difficulties and to determine the interactions between the factors, a response surface methodology (RSM) is employed for the optimization (Hamsaveni et al., 2001; Wang et al., 2009). Response surface methodology which includes factorial design and regression analysis is an effective statistical technique for developing, improving and optimizing of complex processes (Murthy et al., 2000). A great number of successful applications of RSM suggest that second-order relation can reasonably approximate many of the biocatalysis systems (Kalil et al., 2000; Xiong et al., 2004). Right now, RSM has been widely employed to define the relationships between the response and the independent variables in many kinds of chemical and biochemical process optimization.

Optically pure bioactive compounds are preferred over racemic mixtures because they are more target specific and because they show few, if any, undesirable side effects (Wistuba and Schurig, 1992; Ledford and

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Carreira, 1995). As a result, it is expected that the demand for these optically pure compounds will extend in the future (Weijers and De Bont, 1999; Kasai et al., 1998; Kasai and Suzuki, 2003). Enantiomerically pure epoxides such as epichlorohydrin (ECH) can serve as synthons in the preparation of *β*-blockers (Narina and Sudalai, 2007), L-carnitine (Kabat et al., 1997) and radiosensitizer (Hori et al., 1997). Therefore, it has attracted much attention in the development of methods for the synthesis of enantiopure epoxides. Various chemocatalytic and biocatalytic methods have been developed for preparing chiral epoxides (Tokunaga et al., 1997; Swaving and De Bont, 1998; Spelberg et al., 1999, 2004; Li et al., 2006). Among the biocatalytic routes, kinetic resolution of racemic epoxides via an enantioselective hydrolysis reaction by an epoxide hydrolase (EH) might be commercially useful since it is possible to obtain chiral epoxides with high optical purities from relatively cheap and readily available racemates (Santaniello et al., 1992; Shimizu and Katoka, 1999; De Vries and Janssen, 2003). Several attempts for the production of chiral ECH from its racemates by microbial kinetic resolution have been reported. An enantioselective epoxide hydrolase from an actinomycetes source has been observed in a *Nocardia* H8 which showed enantioselectivity in the hydrolysis of racemic ECH (Weijers and De Bont, 1991). Fungal epoxide hydrolysis has been described for *Aspergillus niger* (Choi et al., 1998, 1999) and *Rhodotorula glutinis* (Weijers 1997; Kim et al., 2004; Lee, 2007). However, microbial cells only include small amount of EH and often need to be induced to express them. We have previously described a novel crude EH prepared from domestic duck liver that can enantioselective resolution racemic ECH to produce (S)-enantiomer (Lu DQ, Ling XQ, Wang J, Tu QB, Ouyang PK (2009). Nanjing University of Technology, China, patent application number: 200910032723.9), which is an easier and more economical source of EH.

Up to now, there are several researches about biocatalytic properties of EH from several of microbes (Kim et al., 2004; Lee, 2007). Unfortunately, less attention has been paid to the analysis and optimization of biocatalysis enantioselective production of enantiopure ECH. It is in urgent need of using a favorable method to study the condition in this considerable process. The objective of the present work was to apply statistical methods to optimize reaction conditions for higher optical purity of chiral ECH catalyzed by the novel EH. In the present investigation, optimum parameters of reaction temperature, pH and reaction time were obtained by RSM.

MATERIALS AND METHODS

Preparation of crude EH

Fifty grams of fresh liver prefrozen at 0 - 4℃ was homogenized four times in a Waring blender for 30 s each. The homogenate was poured into 200 ml acetone at -20℃ with stirring. When the suspension had settled, the precipitate was collected by filtration using a Büchner funnel under vacuum, washed with 100 ml cold acetone followed by 50 ml cold ether and dried in refrigerator at 0 - 4℃ (Lu et al., 2006).

Enantioselective hydrolysis of ECH by crude EH

The dry pellets of crude EH (0.6 g) was suspended in 20 ml phosphate buffer (200 mmol/l) in 50 ml screw-cap bottles sealed with a rubber septum and heated in a temperature controlled heating water bath (Taicang, Jiangsu, China). The kinetic resolution was started by adding ECH as substrates. Samples were withdrawn from the reaction mixture periodically and analyzed by gas chromatography immediately.

Experimental design and optimization by response surface methodology (RSM)

A Box-Behnken factorial design with three factors and three levels, including three replicates at the centre point, was used to generate 15 treatment combinations, with reaction temperature, pH and reaction time as variables. The composition of the model was established from these preliminary assays. For statistical calculation, the variables were coded according to (Canettieri et al., 2007). The statistical model was based on the RSM which equation was determined by analysis of linear multiple regression using the statistical software (Statsoft, v 6.0, USA). The enantiomeric excess (e.e.%) of (S)-ECH produced from its racemates catalyzed by the EH from domestic duck liver was taken as the dependent variable or response of the design experiments. The statistical significance of the regression coefficients was determined by t test. The variables were correlated by empirical models.

Enantiomeric excess of chiral ECH (%)

The e.e.% of (S)-ECH was calculated as expressed in Equation (1).

$$\text{Enantioselective excess of (S)-ECH} = \frac{S - R}{S + R} \times 100\% \quad (1)$$

where S and R are the molar concentration of (S)-ECH and (R)-ECH in the reaction sample, respectively.

Analytical method

The gas chromatography analysis were performed using a SP-6890 gas chromatograph (Lunan Ruihong Ltd, Shandong, China) equipped with ZKAT-Chiral B capillary column (20 m × 0.25 mm × 0.5 μm). The injector and flame ionization detector (FID) temperature were 150 and 250℃, respectively. The oven temperature was held at 100℃, N_2 being the carrier gas (1.1 ml/min). The injected volume was 0.4 μl with a split ratio set at 60:1.

RESULTS AND DISCUSSION

Selection of optimum biocatalyst, pH value, reaction teperature and time for enantioselective production of chiral ECH.

The enantioselective resolution of ECH was mainly a chemical process achieved by hydrolysis of epoxy bond. In order to screen out satisfactory EH with the most

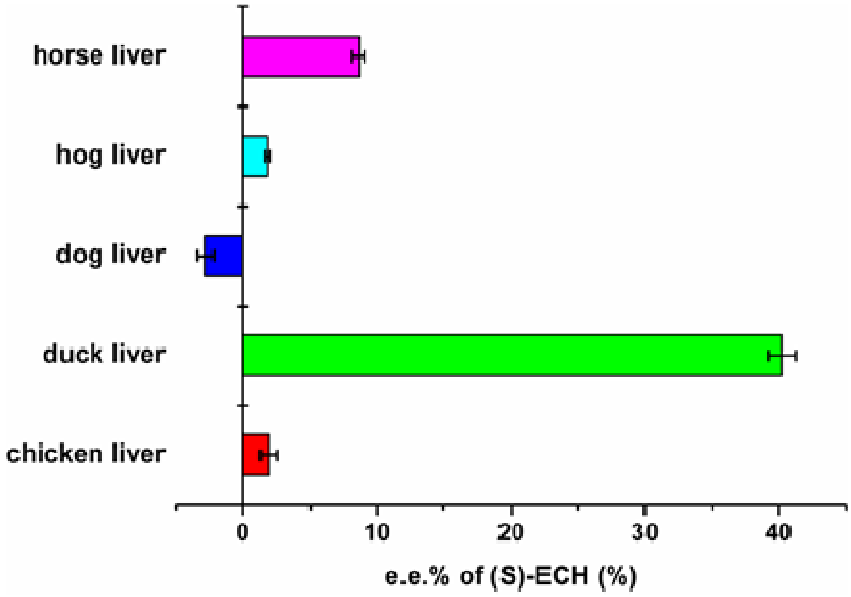

Figure 1. The influence of different liver sources of EH on e.e.% of (S)-ECH.

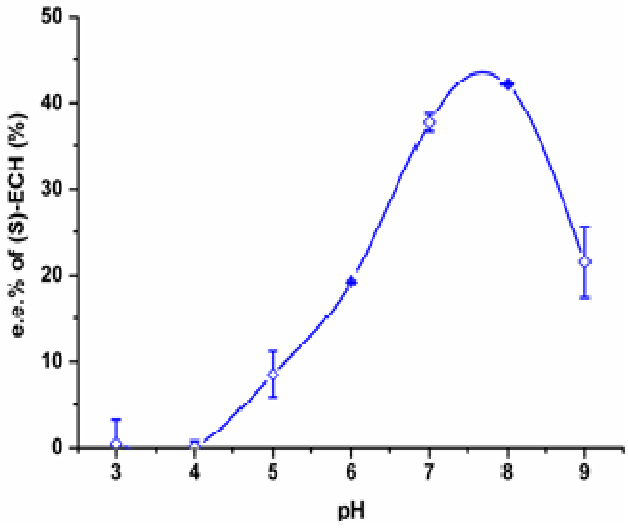

Figure 2. The influence of different pH on e.e.% of (S)-ECH.

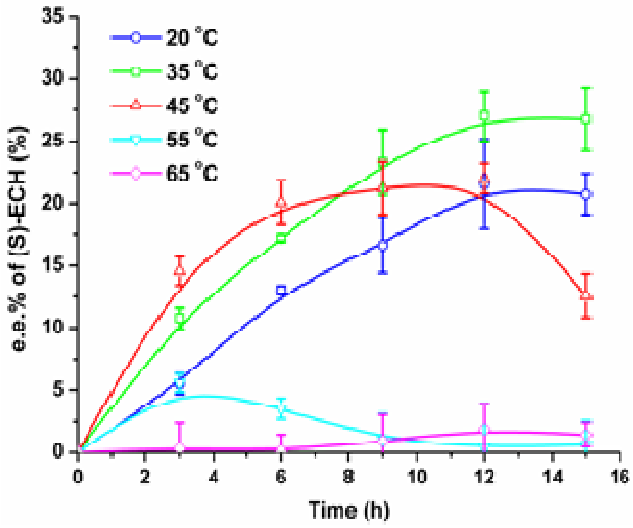

Figure 3. The influence of different reaction temperature and time on e.e.% of (S)-ECH.

preferable enantioselectivity to racemic ECH, five animal liver sources of EH (chicken, duck, dog, hog and horse) were investigated in these experiments. The results indicated that five sources of EH showed certain enantioselectivity to hydrolysis of ECH (Figure 1). Compared with the others, EH from domestic duck liver exhibited higher catalytic enantioselectivity, which indicated that duck liver EH is a preferable biocatalyst that can be used for enantioselective resolution of racemic ECH.

The e.e.% of (S)-ECH increased with pH from 3 to 8 and thereafter decreases between pH 8 to 9 (Figure 2). Therefore, 7-8 was selected the most suitable pH value for the enantioselective production of (S)-ECH.

The concentration of resolution reaction temperature and time influence the e.e.% of (S)-ECH catalyzed by duck liver EH (Figure 3). The highest e.e.% of (S)-ECH is at the resolution reaction temperature of 35℃ and time of 12 h. The curves of e.e.% of (S)-ECH between times at various temperatures indicated that reaction temperature and time are two significant parameters in process of producing (S)-ECH. For the different reaction temperatures (20, 35, 45, 55 and 65℃), the e.e.% of (S)-ECH increased with reaction time and reached the maximal value when the reaction time were 12, 12,10, 3 and 12 h, respectively. And then, the e.e.% of (S)-ECH decreased

Table 1. Coded values of variables used in Box-Behnken experimental design.

No.	Variables	Coded values		
		-1	0	+1
1	pH	6	7	8
2	Temperature (℃)	20	35	50
3	Time (h)	5	10	15

with the increase in the reaction time. It can also be seen from Figure 3, within the temperatures investigated, at the same reaction time such as 6 h, the e.e.% of (S)-ECH increased with the increasing temperature between 20-45℃ and decreased with the increasing temperature between 45-65℃. However, with the increase of reaction time, the spontaneous hydrolysis speed of ECH without enantioselectivity is increases, so selecting optimum hydrolysis temperature and time is essential to obtain maximum e.e.% of (S)-ECH. In the comprehensive consideration of the hydrolysis temperature and time, 20-45℃ and 8-12 min were selected the most suitable hydrolysis temperature and time, respectively, for the production of chiral ECH from its racemates and were used in the subsequent assays.

Regression model of response from Box-Behnken design and RSM strategies

The coded values of independent variables are given in Table 1. 15 experimental runs with different combinations of three factors and three levels were carried out (Table 2). The variables used for the statistical analysis were pH value, reaction temperature and reaction time, named X_1, X_2, X_3 in this design, respectively. The design of experiments through enzyme hydrolysis and the respective experimental and predicted e.e.% of (S)-ECH from its racemates are given in Table 2. It can be seen from Table 2, there was a considerable variation in the e.e.% of (S)-ECH depending on the three chosen variables. The maximum e.e.% (85.62%) was achieved in run number 15, while the minimum e.e.% (0.56%) was observed in run number 4. The maximum value was much higher than the minimum one, which adequately indicated that selecting appropriate reaction conditions would significantly enhance optical purity of (S)-ECH.

Data were analyzed by non-linear multiple regression using statistical software (Statsoft, v. 6.0, USA). After regression analysis, the second-order response model was obtained which is given in equation (2).

$$\text{e.e. of (S)-ECH (\%)} = 84.06 - 3.35X_1 - 30.09X_1^2 - 9.83X_2 - 37.82X_2^2 + 4.45X_3 - 23.40X_3^2 - 9.77X_1X_2 - 21.43X_1X_3 - 19.25X_2X_3 \quad (2)$$

The simple model only considering the significant terms expressed by equation (3) was generated as:

$$\text{e.e. of (S)-ECH (\%)} = 84.06 - 30.09X_1^2 - 37.82X_2^2 - 23.40X_3^2 - 21.43X_1X_3 - 19.25X_2X_3 \quad (3)$$

The results on estimated effects, standard error, *t* test and significance level for the model representing e.e.% of (S)-ECH are presented in Table 3. As could be seen, positive estimate coefficients for X_3 (time) indicated a linear effect to increase e.e.%, while negative coefficient of X_1 (pH) and X_2 (temperature) showed the opposite effect. The *P*-values are used as a tool to evaluate the significance of each of the coefficients. In Table 3, the quadric term of these three variables had a significant effect. Significant interactions existed in X_1 and X_3, X_2 and X_3, but interactions between X_1 and X_2 were found to contribute to the response at an insignificant level.

The analysis of variance (ANOVA) for response surface quadratic model is summarized in Table 4. The model F-value of 13.29 and the low P-value of 0.00082 implied the model was significant. The fits of the model was also expressed by the coefficient of determination R^2, which was found to be 0.9599, indicating that 95.99% of the variability in the response could be explained by the model. Generally, a regression model with an R^2-value higher than 0.9 could be considered as having a very high correlation (Li and Lu, 2005). Therefore, the present R^2-value reflected a very good fit between the observed and predicted responses.

Localization of optimum condition

In order to determine the most adequate operating conditions and to analyze the process of resolution reaction, the response surfaces were plotted using Equation (2) for three possible combinations. The response surface and contour diagrams of e.e.% of (S)-ECH as a function of: pH (X_1) and temperature (X_2), pH (X_1) and time (X_3), temperature (X_2) and time (X_3) are presented in Figures 4, 5 and 6, respectively. The simultaneous analysis of so many plots is a complex task if practical short cuts taking advantage of prior knowledge of the process are not adopted. In Figures 4, 5 and 6, three variables were the main factors that affects e.e.% of (S)-ECH produced by novel epoxide hydrolase hydrolysis of racemic ECH. For example, it can be seen that only moderate pH value, temperature and time in the process of hydrolysis reaction lead to high *e.e.*% of (S)-ECH, while low e.e.% values were obtained at the two terminal of three factors. So these three variables should be selected at an appropriate value range.

The optimum values were found by solving the regression equation analytically (Agarry et al., 2008). The solution was obtained by submitting the levels of the factors into the regression equation (Equation 2). The optimal values of test factors in the coded units were X_1 = - 0.1038, X_2 = - 0.1707 and X_3 = 0.2129. At these values,

Tabel 2. Box-Behnken design matrix with experimental and predicted values of enantiomeric excess of (S)-ECH.

No.	Variables			Enantiomeric excess of (S)-ECH (%)	
	X_1 (pH)	X_2 (Temperature)	X_3 (Time)	Experimental	Predicted
1	- 1	- 1	0	12.20	19.56
2	1	- 1	0	31.81	32.40
3	- 1	1	0	20.03	19.45
4	1	1	0	0.56	- 6.80
5	- 1	0	- 1	18.17	8.05
6	1	0	- 1	47.55	44.20
7	- 1	0	1	56.46	59.81
8	1	0	1	0.13	10.25
9	0	- 1	- 1	6.21	8.97
10	0	1	- 1	17.10	27.81
11	0	- 1	1	67.08	56.37
12	0	1	1	0.99	-1.78
13	0	0	0	84.27	84.06
14	0	0	0	82.31	84.06
15	0	0	0	85.62	84.06

Table 3. Regression coefficients and their significances for enantiomeric excess of (S)-ECH from the results of Box-Behnken experimental design.

Model term	Estimate	Degree of freedom	Standard error	*t*-value	P-value
Intercept	84.06	1	6.25	13.44	< 0.0001
X_1	- 3.35	1	3.83	- 0.88	0.42
X_1^2	- 30.09	1	5.64	-5.34	0.0031
X_2	- 9.83	1	3.83	- 2.57	0.050
X_2^2	- 37.82	1	5.64	- 6.71	0.0011
X_3	4.45	1	3.83	1.16	0.30
X_3^2	- 23.40	1	5.64	- 4.15	0.0089
X_1X_2	- 9.77	1	5.42	- 1.80	0.13
X_1X_3	- 21.43	1	5.42	- 3.96	0.011
X_2X_3	- 19.25	1	5.42	- 3.55	0.016

Table 4. Variance analysis of regression equation.

Source	Sum of squares	Degree of freedom	Mean square	F-value	P-value
Model	14030.95	9	1558.99	13.29	0.00082
Residual	586.52	5	117.30		
Lack of fit	580.98	3	193.66	69.99	0.014
Pure error	5.53	2	2.77		
Total	14617.47	14			

R-Squared = 0.9599, Adjusted R-Squared = 0.8877.

actual reaction conditions were determined as followed: pH 7.1, temperature, 32.4℃ and time 11.1 h. The maximum predicted value of e.e.% of (S)-ECH was 85.55%.

Model adequacy checking

In order to check approximation between the fitted model

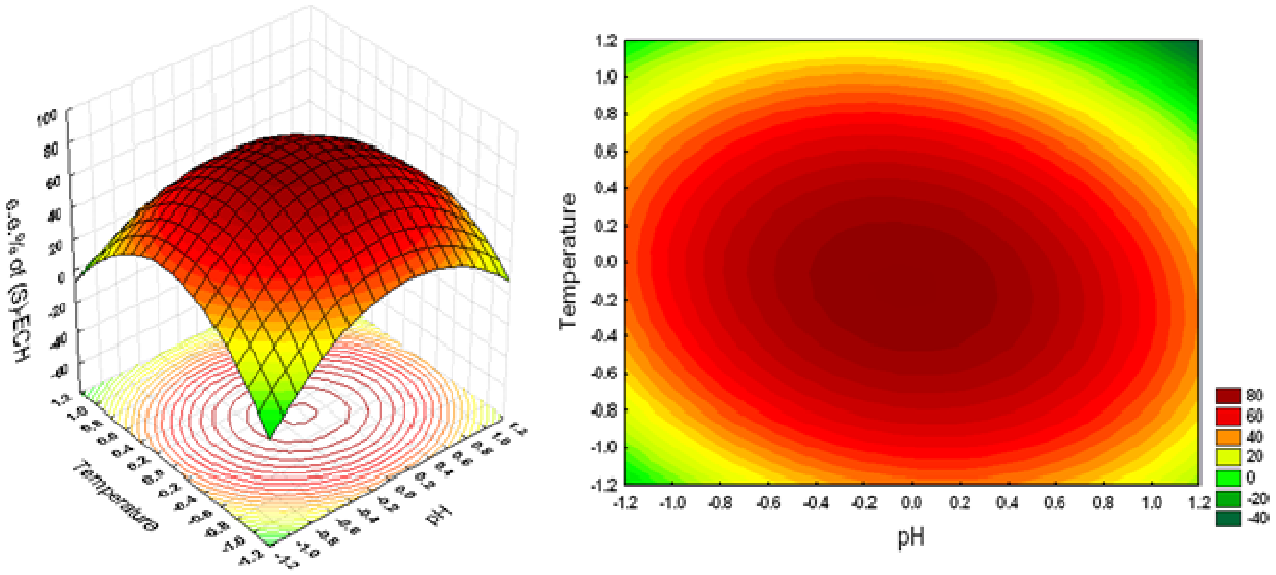

Figure 4. Response surface and contour diagrams of the combined effects of pH (X_1) and temperature (X_2) on enantiomeric excess of (S)-ECH.

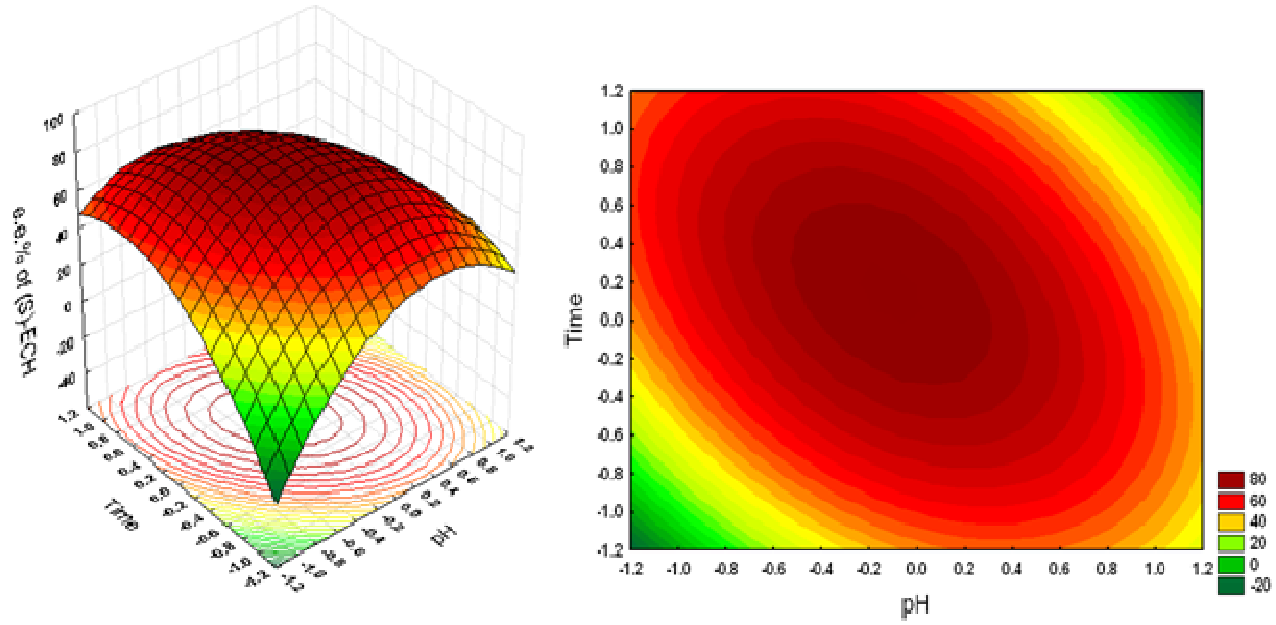

Figure 5. Response surface and contour diagrams of the combined effects of pH (X_1) and time (X_3) on enantiomeric excess of (S)-ECH.

and actual biocatalysis system, the residuals from the least squares fit were investigated to judge model adequacy. The plots of residuals versus the predicted values were depicted as in Figure 7. The residuals were distributed randomly on the display, indicating that the variance of the original experimental results was constant for all values of responses, which was preferable based on the judge of adequacy. As a result, it can be concluded that this fitted model is adequate to reveal the bio-catalytic characteristics of the novel epoxide hydrolase by RSM.

Experimental validation of the optimized condition

To confirm the model adequacy for predicting the maximum enantioselective excess of (S)-ECH, three

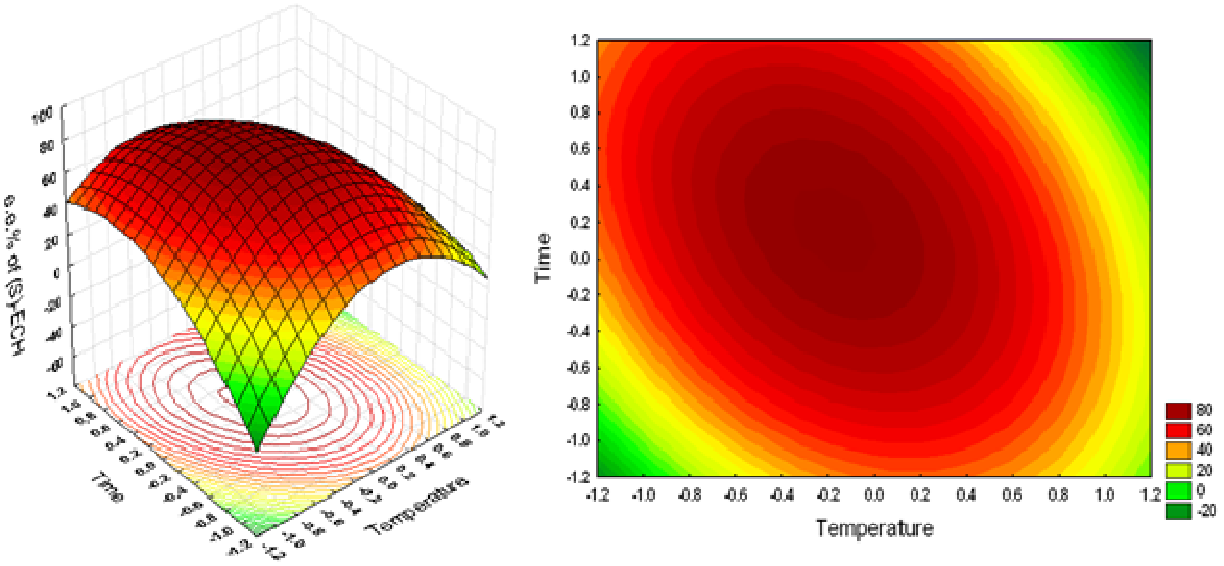

Figure 6. Response surface and contour diagrams of the combined effects of temperature (X_2) and time (X_3) on enantiomeric excess of (S)-ECH.

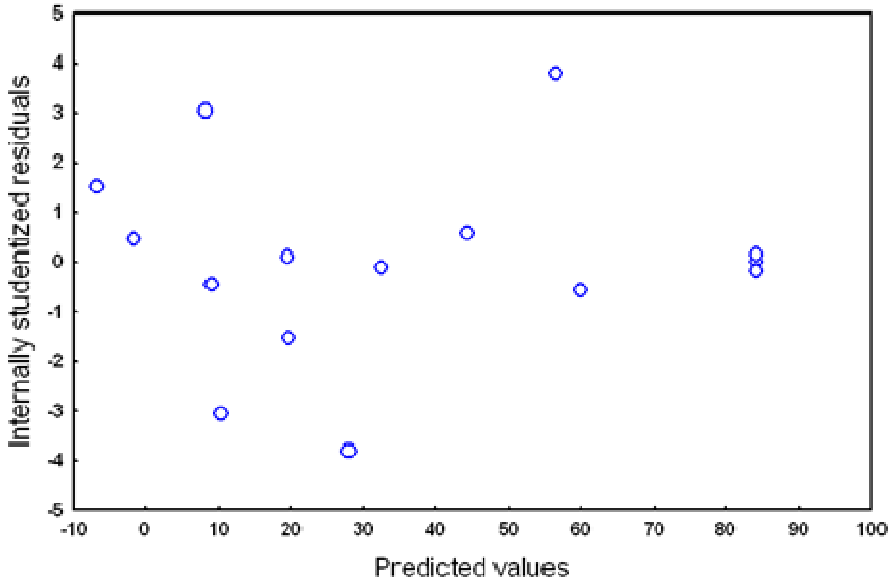

Figure 7. Plot of Internally studentized residuals versus predicted values.

additional experiments under this optimum reaction condition were performed. The mean value of e.e.% of (S)-ECH was 86.14%, which was in excellent agreement with the predicted value (85.55%). The agreement between predicted value and experimental value of e.e.% of (S)-ECH confirmed the significance of the model.

Conclusion

A novel epoxide hydrolase from liver of domestic duck was utilized to catalyze enantioselective hydrolysis reaction of racemic ECH and its reaction process conditions were successfully achieved by RSM in this study. Three hydrolysis reaction parameters for production of (S)-ECH were optimized by using Box-Behnken design of RSM: pH value 7.1, reaction temperature 32.4℃ and reaction time 11.1 min. The e.e. of (S)-ECH after optimization was 86.14% while predicted value was 85.55%. RSM as an effective method proved to be quite adequate for the design and optimization of the process of enantioselective production of (S)-ECH from its racemates and

the developed production process indicated the novel epoxide hydrolase from domestic duck liver was a preferable biocatalyst that can be used for enantioselective resolution of racemic ECH.

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