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Baohuoside I production through enzyme hydrolysis and parameter optimization by using response surface and subset selection

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ABSTRACT

A rapid and efficient baohuoside I preparation method was established. A uniform design coupled with subset selection was employed to determine pH, reaction time, and temperature parameters, as well as dextranase hydrolysis efficiency. Hydrolysis parameters were optimized using response surface and subset selection. Our results showed that pH plays an important role in the hydrolysis reaction within a relatively narrow range (pH 4–7). Temperature was the secondary factor, which was positively correlated with conversion rate. A 3-h reaction time was sufficient. Finally, a relatively good hydrolysis parameters were found, and their effectiveness was verified.

1. Introduction

In China, the Epimedii herb (Yinyanghuo, YYH) is one of the most frequently used tonic herbs prescribed to treat osteoporosis, delay aging, and improve sexual function [1–4]. Recently, YYH and its effect on bone healing was studied [5,6], and the possible mechanism of its primary active compounds was investigated [1,7,8]. Among these bioactive components, baohuoside I, a trace component, was found to inhibit osteoclasts [9], hypoxia-inducible factor-1α in human osteosarcoma cells [10], melanogenesis [11], MCF-7 cells [12], and PC-3 prostate cancer cells [13]. Though it is a highly active compound, research on baohuoside I is relatively insufficient because of its trace quantities in YYH. Thus, the methodology to prepare this trace component is important for further pharmacological studies. It is well known that baohuoside I is a metabolite of icariin. Fig. 1 shows the relationship between baohuoside I and icariin. Recently, studies on preparing baohuoside I have been conducted, including acid/base/enzymatic hydrolysis of icariin [14,15] and column chromatography [16]. However, all of these methods require long reaction time or complicated processes, and more importantly, the acid or base conditions could weaken the activity of baohuoside I. Therefore, it is necessary to optimize the parameters to improve icariin hydrolysis efficiency while maintaining baohuoside I activity.

Uniform design (UD), first proposed by Wang and Fang [17,18], seeks design points that are uniformly scattered on the domain. Compared to other experimental design methods, such as central composite [19,20] and orthogonal design [21], UD predominates by requiring fewer test numbers without loss of information [22,23]. The effect of the interaction of different factors and important index of factors could be analyzed by a second-order polynomial regression without a mono-factor experiment.

This is the first report of the preparation of baohuoside I by dextranase hydrolysis of icariin and optimized reaction parameters of UD coupled with subset selection and response surface methodology (RSM) [24–26]. A high production rate could be achieved under moderate condition. This methodology of baohuoside I production unsing dextranase could be used as an industry standard.

2. Experimental

2.1. Chemicals

Baohuoside I standard (purity ≥ 99%) was purchased from Shanghai Winherb Medical Science Co. (Shanghai, China). Citric acid and sodium citrate were purchased from Shenyang Chemical Reagent Co. (Shengyang, China). Methanol was obtained from Yuwang Industrial Co. (Yucheng, China). Water was of HPLC grade (purified using a Milli-Q gradient purification system; Millipore, MA, USA). All other common chemicals were of HPLC grade and were purchased from Sigma–Aldrich (USA).

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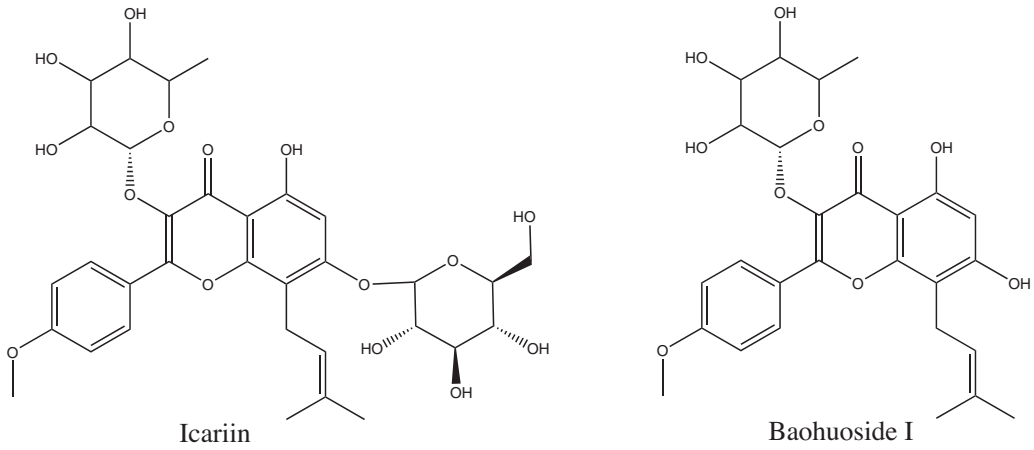

Fig. 1. Structural relation between icariin and baohuoside I. All belong to the flavonoid glycoside family. Icartiin comprises of baohuoside I and glucose through a beta-1, 3-glycoside linkage at the 7′ C—OH site.

2.2. Materials

Icariin was prepared in a homemade preparative chromatography system, and its purity was greater than 98% as determined by HPLC. The preparative chromatography system composed of a Waters DP400 separation system and a Chromatorex C18 preparative column (250 mm × 20 mm, 10 μm). The water bath was obtained from Yuhua Instrument Co. (Beijing, China). Dextranase (10,000 U/mg) was kindly supplied by Yuguang Du, Natural Products and Glyco-Bitotechnology, Dalian Institute of Chemical Physics, CAS, China.

2.3. Preliminary experiments

Initially, the following 5 factors were considered for analysis: temperature, pH, reaction time, Cu^{2+}, and a nonaqueous system.

Cu^{2+} was used at 6.4 μg/ml, whereas ethyl acetate, butanol, and n-hexane were employed in the nonaqueous system to test for hydrolysis. For the other parameters, 2 tests (marked by S1 and S2) were used to evaluate pH, temperature, and reaction time for hydrolysis. S1 and S2 parameters were as follows: S1 – temperature, 50 °C; pH, 3; and reaction time, 1 h (indicated as T50 °C-pH3-RT1 h) and S2 – T40 °C-pH5.4-RT3 h.

2.4. Sample and buffer solution preparation

The different pH buffer solutions were prepared by mixing 0.1 M citric acid and 0.1 M sodium citrate solution at different volumes and measuring the pH. Briefly, buffer solutions with pH values of 2.0, 3.0, 4.2, 5.4, 6.6, and 8.0 were prepared by mixing 10.00, 9.30, 6.15, 3.20, 0.70, and 0 ml citric acid with 0, 0.70, 3.85, 6.80, 9.30, and 10.00 ml sodium citrate, respectively. A dextranase stock solution was prepared by dissolving 4 mg dextranase in 800 μl ultrapure water and stored at 4 °C prior to testing. A 100-μl aliquot of dextranase stock was added to each test solution. For icariin, 1 mg/ml icariin solution in methanol was prepared; next, 1 ml solution was drawn and then dried to obtain 1 mg icariin. In each test solution, 1 mg icariin was added as the substrate.

2.5. Experiment design

UD was introduced to optimize the pH, temperature, and reaction time parameters to achieve the greatest hydrolysis efficiency. A $U_6(6^3)$ UD table (Table 1) was selected for the experimental design, which represented a three-factor-six-level experimental model. All of those required 6 experiments are listed in Table 2 (UD plus a number indicated the corresponding test).

Table 1
$U_6(6^3)$ uniform design table.

Testes		1	2	3	4	5	6
Factors Levels	1	3	6	2	5	4	1
	2	5	3	1	6	2	4
	3	6	5	4	3	1	2

Table 2
Uniform design test table of each factor.

Testes		UD1	UD2	UD3	UD4	UD5	UD6
Factors	Temperature (°C)	40	80	30	60	50	19
	PH (ml)	6.6	4.2	2	8	3	5.4
	Reaction time (h)	6	4	3	2	0.5	1

2.6. Isolation of icariin hydrolysate

After hydrolysis, the hydrolysate was centrifuged at 3000 rpm for 5 min, and the supernatant was discarded. The precipitate was dissolved with 5 ml methanol, sonicated for 5 min, centrifuged at 3000 rpm for 5 min, and the supernatant was collected. This procedure was repeated until no yellow precipitate was found (2 rounds were sufficient). Thus, icariin hydrolysate was separated by dextranase and was ready for analysis.

2.7. HPLC-DAD conditions

Analysis of icariin, baohuoside I, and the hydrolysate were carried out on a Waters 2690 HPLC system consisting of an AutoSampler, Column Compartment, and Photodiode Array Detector. A Hypersil C18 column (250 mm × 4.6 mm, 5 μm; Elite Analytical Instrument Co., Ltd.) was selected to analyze icariin and baohuoside I.

Chromatographic separation was conducted using a mixture of 2 eluents: solvent A, acetonitrile; solvent B, deionized water. A linear gradient program was used as follows: 0–14 min from A/B (25: 75) to A/B (32: 68), and 14–42 min up to A/B (70: 30). The mobile phase was equilibrated at an initial ratio for 30 min before injection. The flow rate was set to 0.8 ml/min, and the detector was set at 270 nm at room temperature. For hydrolysate analysis, 10 μl sample was injected into the HPLC.

2.8. Subsets selection

The conversion rate reflects the capacity to which dextranase can hydrolyze icariin to baohuoside I under different hydrolysis conditions, which could be calculated by Eq. (1).

$$CR = \frac{(Area_{BAO}/514.52)}{(Area_{BAO}/514.52) + (Area_{ICA}/676.77)} \times 100\% \quad (1)$$

CR indicates the conversion rate, $Area_{BAO}$ and $Area_{ICA}$ represent the peak area of baohuoside I and icariin at 270 nm, respectively. The molecular weights of baohuoside I and icariin were 514.52 and 676.77, respectively.

A model of the conversion rate (dependent variable) and 3 factors (independent variable) was built by a second-order polynomial regression (Eq. (2)). Calibration data integrates the UD data, S1, and S2 together and formed a good model. The best subset was obtained by modeling the dependent and independent variables in a second-order polynomial regression.

$$y = b_0 + \sum_{i=1}^{3} b_i x_i + \sum_{i=1}^{3} b_{ii} x_i^2 + \sum_{i,j=1(i \neq j)}^{3} b_{ij} x_i x_j \quad (2)$$

where y indicates the conversion rate, b_0 is the constant coefficient, b_i is the linear coefficient, b_{ii} and b_{ij} are quadratic coefficients, and x_i and x_j represent factors levels, especially b_{ij}, which reflects the interaction of factor i and j. Considering 3 factors, all the 10 regression coefficients and our 8 observations (8 UD testes and 2 preliminary testes), the maximum size of the subset was set to 5 to avoid overfilling. Prediction error sum of square (PRESS), adjusted R-square statistic (adj$R$), Bayesian information criterion (BIC), residual sum of square (RSS) based on leave one out cross validation (LOOCV) [27,28], and the p-value of model were used to evaluate the model. The detailed best subset selecting procedure is described as follows:

(1) Calculate all the possible subsets (constant are always included in the model for accurate parameter estimation).
(2) Fit all the subset and compute PRESS, adj R, BIC, RSS and p-value of each model.
(3) Select the latent subset according to the rule that adj$R$ is larger than 0.85 and RSS is less than 2.
(4) Integrate PRESS and BIC to obtain the best subset from latent subset.

Computation of the best subset was analyzed through MATLAB 7.5, and a matlab code was developed for this purpose. One-way analysis of variance (ANOVA) was preformed using the SPSS 16.0 statistical package for Windows (SPSS Inc., Chicago, IL)

3. Results and discussion

3.1. Preliminary experiments for parameter selection

The purpose of the preliminary experiments was to determine the main factors involved in hydrolysis. Among the factors considered, Cu^{2+} was reported to be an activator to dextranase [29]. However, in our experiment, the test reflected that Cu^{2+} had no significant effect on hydrolysis at 6.4 μg/ml. Previous studies suggested that a nonaqueous system increased enzyme specificity and stability [30,31]. The results indicate that dextranase was a poor hydrolyzer and required long reaction times (longer than 12 h). Furthermore, it was difficult to separate dextranase from baohuoside I due to their low aqueous solubility. Therefore, nonaqueous system and Cu^{2+} were not investigated further. The results showed that S2 gave a higher conversion rate (98.51%) than S1 (1.11%). Therefore, the factor levels were set to the S2 parameters in UD.

Table 3
Peak area of icariin, baohuoside I, and the conversion rate of UD testes and preliminary experiments.

Test	Icariin	Baohuoside I	CR
S1	6076571.5	67921.5	0.0145
S2	420533.9	27811794	0.9886
UD1	1736206	54940736	0.9765
UD2	20492019	25212670	0.6180
UD3	72075574	713898.15	0.0129
UD4	71298452	628861.91	0.0115
UD5	58308930	1082583.6	0.0238
UD6	44427147	26224922	0.4370

3.2. HPLC profile of icariin, baohuoside I and hydrolysate from icariin

Hydrolysate from icariin was identified by HPLC with DAD detector at 270 nm. Fig. 2 indicates the chromatographic profile of icariin and the baohuoside I standard. The retention times of icariin and baohuoside I were approximately 19.03 min and 27.62 min, respectively. Fig. 3 shows the chromatographs of 6 UD testes and 2 preliminary experimental results. Icariin and baohuoside I were identified by comparing to their standards. From the chromatographic profile, UD1 and S2 represented maximum dextranase activity, UD2 and UD6 suggested mid-level activity, and the remainder had poor activity.

3.3. Conversion rate calculation

The peak area was calculated by automatic integration with the *Peak With* set to 30 and the *Threshold* set to 50; the conversion rate was calculated according to Eq. (1). Table 3 represents the peak areas of icariin and baohuoside I, and the conversion rate of the UD tests and preliminary experiments. UD3 and UD4 showed that dextranase is sensitive to pH, and it may be denatured by weakly base or strongly acidic hydrolysis conditions. This can be proved by comparing UD3 with S2. The hydrolytic efficiency was entirely different when the reaction times and temperatures were similar between UD3 and S2. Therefore, pH is an important factor for hydrolysis, with the optimal pH being around 4–7. UD2 and UD6 both were reactive at the optimal pH; however, they were not important when suboptimal temperature and reaction times were used. Nevertheless, they still achieved a moderate rate of conversion (61.80% for UD2 and 59.02% for UD6), which implied that these 2 factors were not as critical as pH.

We used the conversion rate for evaluating enzyme activity. Compared to the yield rate, the conversion rate is a more reasonable parameter. Since the yield rate is related to sample preparation, hydrolysis, and sample collection, most aspects of the experimental procedure could affect the yield rate. However, the conversion rate is related only to the enzyme activity.

3.4. Model fitting

A total of 255 subsets were obtained for a conditioned combination of 5 out of 10 factors (always constant in a given model). Fig. 4A is the model adequacy checking scatter plot grouped by the p-value. Since the big difference between models in terms of the RSS, the models with small RSS were compressed into a narrow strip. However, only the model with a large adj$R$ and a small RSS represents a latent model, which is located in the lower right panel in Fig. 4A; therefore, we enlarged this section in Fig. 4B.

According to the rule mentioned above, all latent subsets with their adj$R$, PRESS, BIC, RSS, and p-value together were obtained (Table 4). Since subset 3, 5, 6, and 7 were not statistically significant, they were excluded first. Subset 4 was also excluded as it only

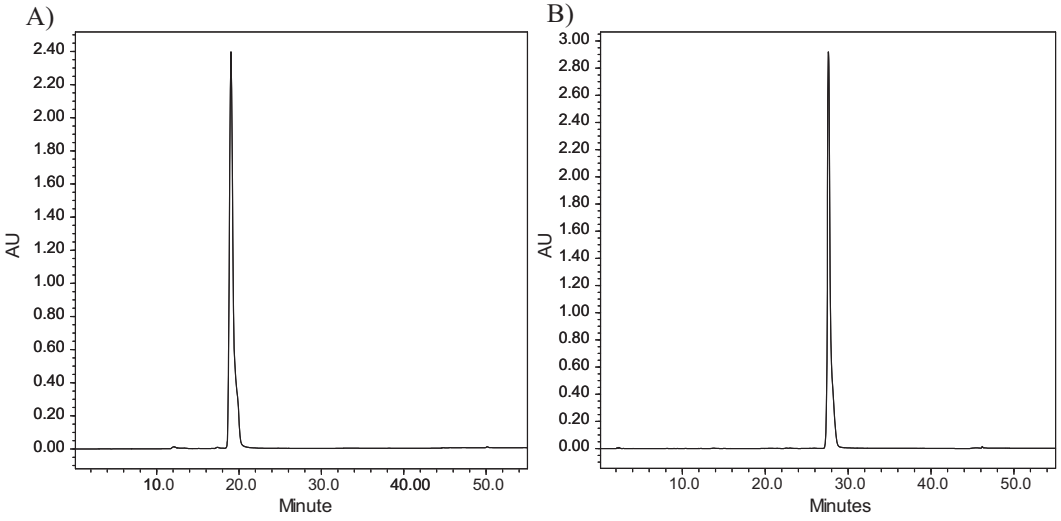

Fig. 2. Chromatographic profile of (A) icariin and (B) baohuoside I.

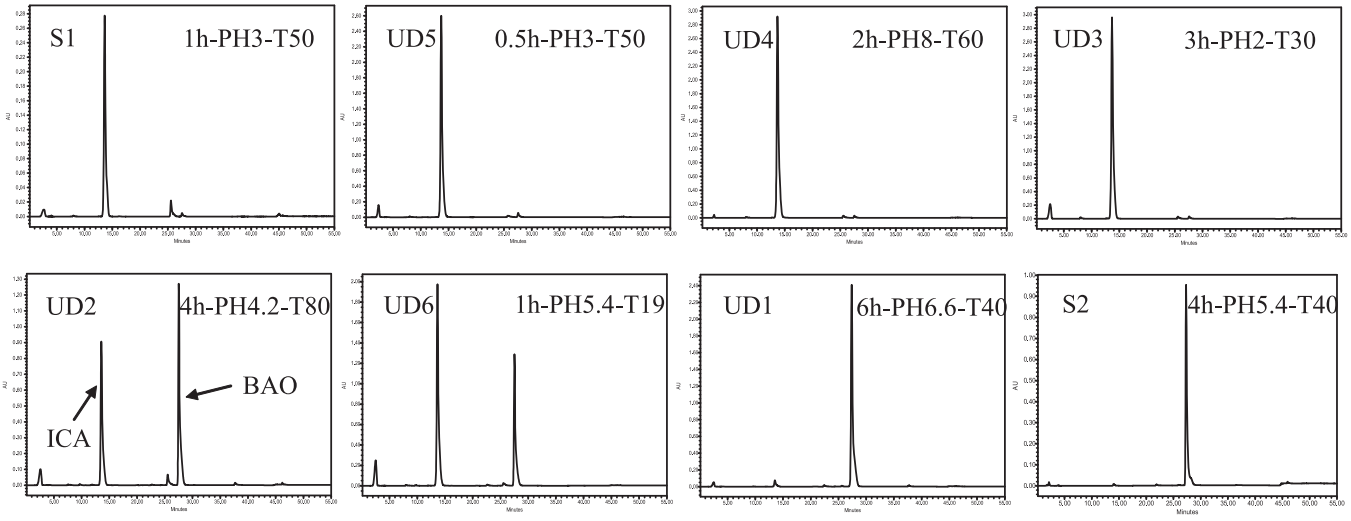

Fig. 3. Chromatographic profile of the hydrolysis result under various conditions.

contained 2 factors, leading to an underestimation. Subset 2 was selected as the best subset for the smaller BIC, PRESS, *p*-value, and bigger adj*R* compared to subset 1, although the RSS was relatively large compared to subset 1. Especially, choosing the best model is not always a robot method for avoiding overfilling in LOO cross validation [32,33]. Compared with other subsets, the BIC of subset 2 was −19.1312, which reflected that subset 2 is a concise model when it involves RSS and the number of selected variables. The adj*R*,

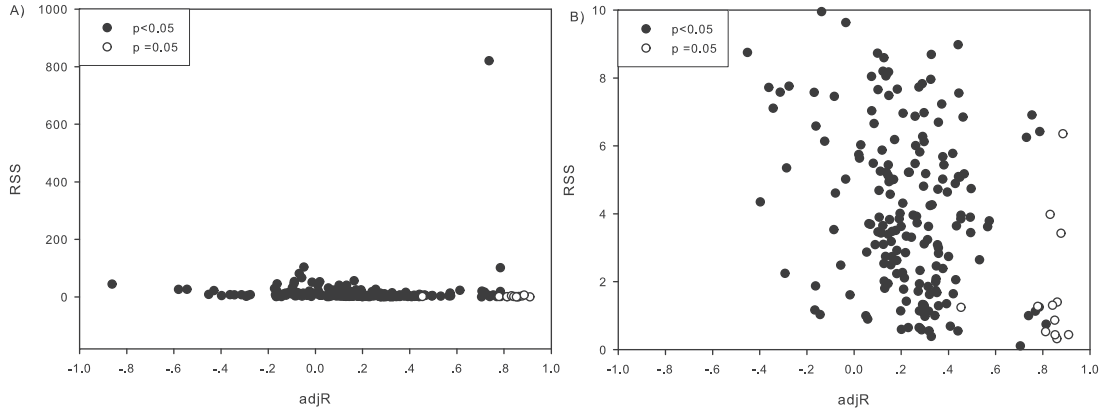

Fig. 4. Adequacy checking plot. (A) Scatter plot of adj*R* vs. RSS grouped by *p*-value. (B) Enlarged plot of (A) in the lower right panel.

Table 4
The latent subset and the corresponding adj$R$, PRESS, BIC, and RSS.

Number	Latent Subset	adj$R$	PRESS	BIC	RSS	P
1	$x_2, x_3, x_1^2, x_2^2, x_1x_2$	0.9514	2.2456	−16.7022	0.1886	0.0344
2	$x_2, x_1^2, x_2^2, x_1x_2, x_1x_3$	0.9758	0.5607	−19.1312	0.4070	0.0172
3	$x_2^2, x_3^2, x_1x_2, x_1x_3, x_1x_3$	0.8829	0.7754	−13.6464	0.1632	0.0816
4	x_1, x_3, x_1^2, x_1x_3	0.9066	0.2487	−13.9262	0.2900	0.0195
5	$x_1, x_2, x^2, x_1x_2, x_2x_3$	0.9257	15.0644	−15.2281	1.1398	0.0522
6	$x_1, x_3, x_1^2, x_3^2, x_1x_3$	0.8641	0.9480	−13.1299	1.4975	0.0943
7	$x_1, x_3^2, x_1x_2, x_1x_3, x_2x_3$	0.8891	0.7975	−13.8369	1.4000	0.0773

Table 5
Analysis of variance of selected model (modeled by subset 2).

Model		Sum of squares	*df*	Mean Square	*F*	Sig.
Subset 2	Regression	1.274	5	0.255	57.558	0.017
	Residual	0.009	2	0.004		
	Total	1.283	7			

PRESS, BIC, and RSS of subset 2 was 0.9758, 0.5607, −19.1312, and 0.4070, respectively, and the selected variables were expressed as $[x_2, x_1^2, x_2^2, x_1x_2, x_1x_3]$. The final equation was as follows:

$$y = -1.7380 + 0.9608 \times x_2 - 0.0005 \times x_1^2 - 0.1288 \times x_2^2 + 0.0076 \times x_1x_2 + 0.0033 \times x_1x_3$$

In the equation, x_1, x_2, x_3, and y represents temperature, pH, reaction time, and conversion rate, respectively. The standardized regression coefficient for the equation were 4.552, −2.128, −6.128, 2.433, and 0.810, which represented the indices used to evaluate the importance of the conversion rate to some extent. Therefore, of the 3 factors, pH is considered the most influential on dextranase activity. The negative coefficients of $x_2 \times x_2$ and $x_1 \times x_1$ suggests that they can achieve the maximum conversion rates at a proper pH and temperature. There are also some interactions between temperature and pH or reaction time. This implies that the hydrolysis efficiency will be maximized under optimal parameters.

The analysis of variance (Table 5) was the adequacy and significance analysis of the selected model based on the subset 2. The result indicated that the model selected was statistically significant at $p = 0.05$. Table 6 displays a more detailed outcome of the estimation of the regression coefficient. All variables in the best subset were statistically significant, with p-values between 0.009 and 0.038. This demonstrates that the selected best subset is significant.

3.5. Response surface and optimization

Response surfaces were generated by fixing one factor and plotting others against the conversion rate. Fig. 5A shows the effects of pH and temperature on the conversion rate at a reaction time of 4 h. This figure indicates that an excellent conversion rate can be achieved when the pH ranges from 4 to 6 (i.e. S2 and UD1), otherwise, the conversion rate drops dramatically (i.e., UD3 and UD4). For temperature, it is parallel to the long axis of the contour plot in Fig. 5A, which shows a small impact of temperature on the activity compared to pH. A weak interaction was also detected with low temperature and small pH increments. Fig. 5B reflects the relationship of reaction time and pH to the conversion rate at 45 °C. If the pH is relatively stable, the conversion rate could slightly alter when the reaction time changes from 0 to 7 h (i.e., S2 and UD1). This indicates that reaction time is a minor factor for conversion rate. A long reaction time is essential for complete hydrolysis, all of which depends on the goal of the experiment. Temperature is a moderate factor from Fig. 5C; however, it means that we can

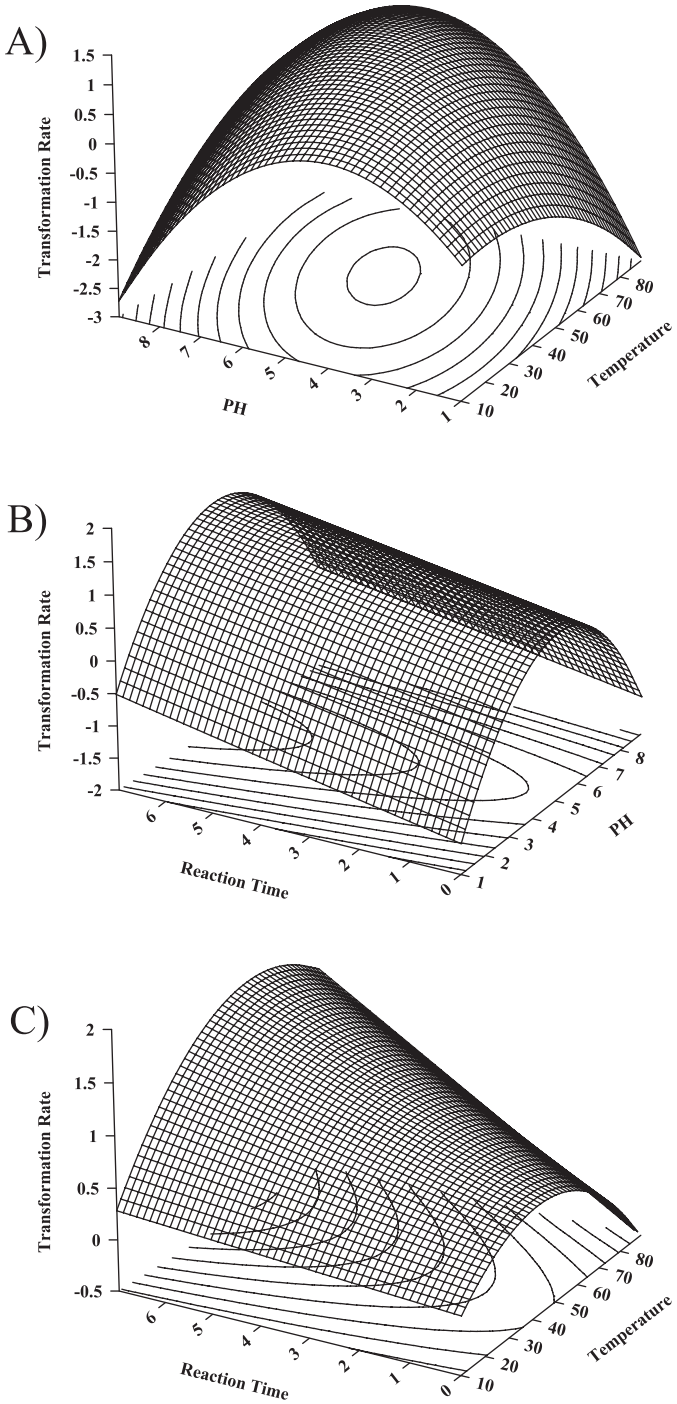

Fig. 5. Response surface on conversion rate and three influential factors: (A) temperature and pH at a reaction time equal to 4 h; (B) pH and reaction time at a temperature equal to 40 °C; (C) temperature and reaction time at a pH equal to 5.4.

Table 6
Regression coefficients estimates of subset 2 using for modeling.

Model		Unstandardized coefficients		Standardized coefficients	*t* value	Sig.
	B	Std. Error	Beta			
Second-order regression	(Constant)	−1.738	0.193		−9.023	0.012
	x_2	0.961	0.093	4.552	10.338	0.009
	${x_1}^2$	0.000	0.000	−2.128	−6.755	0.021
	x_2^2	−0.129	0.015	−6.128	−8.600	0.013
	x_1x_2	0.008	0.002	2.433	5.000	0.038
	x_1x_3	0.003	0.000	0.810	9.540	0.011

Table 7
Comparison of optimum parameters from 3 enzymes for icariin hydrolysis.

	Time/h	Temperament/°C	pH	Ratio	CR (%)
Beta-glucosidase	5	40	6	1	87.7
Cellulsase	48	50	5.2	1	95.37
Dextranase	3	40	5.4	2	98

obtain a relatively stable conversion rate in a wide range from 20 °C to 80 °C. UD2 and UD6 serve as strong proof of that concept. The optimal temperature is around 40–60 °C, as shown in Fig. 5B. Fig. 5A indicates that the optimal pH is approximately 4–6. In our experiment, we set the optimum pH to 5.4, because it was located in the middle of the range and was easy to obtain (according to Table 1). High temperature adversely affected dextranase stability and required a heating apparatus, therefore, the optimum temperature was room temperature. In terms of reaction time, 3 h was sufficient to achieve a 98.86% conversion rate at S2, which was sufficient in most cases. For an enzyme reaction, the reaction rate dramatically decreases when the ratio of product to starting material increases; therefore, it may not be worth prolonging the reaction time to achieve a higher conversion rate.

Finally, we tested the conversion rate at room temperature (nearly 22 °C), pH 5.4, and at a reaction time of 3 h, which resulted in a 93.8% conversion rate. Although the conversion rate was smaller than UD1 and S2, we lowered the temperature to 22 °C. This parameters condition is moderate and probably suitable for industry production. Furthemore, the purity of baohuoside I prepared under this condition meets the demand of many experiments.

3.6. Comparison to other enzyme used for baohuoside I production

The hydrolysis ability of dextranase was also compared to that of beta-glucosidase [14] and cellulase [34], which were used also for preparing baohuoside I in some research articles. The optimum parameters for the three enzymes used for for icariin hydrolysis are listed in Table 7. Obviously, at the optimum parameters, dextranase displays a shorter reaction time and higher conversion rate. Additionally, the ratio of icariin to dextranase is larger than that with beta-glucosidase and cellulase, which explains the high enzyme activity.

4. Conclusion

We used subset selection and response surface methodology to obtain optimum conditions at pH 5.4, a temperature of 22 °C (room temperature), and a hydrolysis time of 3 h with a conversion rate of approximately 94%. When the temperature is up to 40 °C (heating apparatus needed), the conversion rate reaches 98%. Compared to beta-glucosidase and cellulase, dextranase works in a milder environment and hydrolyzes more efficiently. Compared with acid/base hydrolysis, the dextranase method could reduce the loss of baohuoside I. As the dextranase is not particularly sensitive to temperature, even at room temperature, it still has high activity with a conversion rate of 93.8%. Therefore, it has great potential in industrial production. Additionally, subset selection is an effective method for small samples, especially in experimental design. A concise and significant model can be built based on cross-validation and checking the model adequacy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.01.017.

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