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Response surface optimization of ultrasound-assisted enzymatic extraction polysaccharides from *Lycium barbarum*

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ABSTRACT

In this study, an efficient ultrasound-assisted enzymatic extraction procedure for the water-soluble polysaccharides from the fruit of *Lycium barbarum* was investigated and optimized. Response surface methodology (RSM) based on a three-level four-factor Box Behnken Design (BBD) was employed to optimize the extraction conditions inlcluding extraction time, ultrasonic output power, cellulose concentration and extraction temperature. The experimental data were adequately fitted into a second-order polynomial model. The optimized conditions were as follows: extraction time 20.29 min, ultrasonic output power 78.6 W, cellulose concentration 2.15%, extraction temperature 55.79 °C. Under these conditions, the experimental yield of polysaccharides was 6.31 ± 0.03%, which matched with the predictive yield of 6.32% well.

1. Introduction

Fruit from *Lycium barbarum* in the family of Solanaceae has been used as a kind of traditional Chinese herbal medicine for thousands of years. Nowadays it is widely used as popular functional food (Li, Li, & Zhou, 2007). Modern studies indicated that polysaccharides from *L. barbarum* have a range of biologic activities, including enhancing immunity, protecting hepatic function, reducing blood glucose, reducing myocardial injury, antivirus, anti-tumor and anti-aging (Chan et al., 2007; Wang et al., 2010). In order to realize all its promising applications, there is an increase in demand to supply the market with high-quality *Lycium barbarum* polysaccharides (LBP). Although there were many researches about biological activity of LBP, little attention was paid to the extraction of LBP.

There are many factors that can affect the yield of LBP, such as the different types of *L. barbarum* and the different extraction methods et al. Among these factors, the relation of the types of *L. barbarum* to the polysaccharides content was quite intimate. Inferior raw material always leads to lower polysaccharides content (Ye, 2009). We selected inferior *L. barbarum* in our study due to considering the industrial production of LBP.

Enzyme-assisted extraction, which is considered as a mild, efficient and environmental friendly extraction method, has been used in the extraction of various kinds of compounds recently (Li, Smith, & Hossain, 2006; Moura et al., 2008; Santamaria et al., 2000). The addition of specific enzymes such as celluloses and proteases during the extraction process can promote the release of intracellular contents by breaking the cell wall and lipid bodies (Moura et al., 2008). Compared with conventional extraction procedures, Enzyme-assisted extraction method has some advantages such as mild extraction condition and a usually higher extraction yield.

Compared with the conventional extraction method, ultrasound-assisted extraction (UAE), which combines ultrasound and traditional solvent extraction, has been proved to improve the extraction efficiency by increasing the yield and shortening the extraction time (Chua et al., 2009; Dudiey Thompson, 1955; Romdhane, 2002). Recently, many researches were conducted to indicate the application of ultrasound in diverse extraction processes (Chen et al., 2007; Chua et al., 2009; Rodríguez-González, Femenia, Minjares-Fuentes, & González-Laredo, 2012). Moreover, previous studies have shown that the introduction of ultrasonic energy during enzymatic treatment have resulted in significant improvement (Yachmenev Val, Blanchard Eugene, & Lambert Allan, 1998; Zhang, Fu, & Liang, 2008). So, the combinational usage of enzyme and UAE during the extraction process would be more effective probably. Up to our best knowledge, this method has not been reported in plant materials extraction previously.

Alternatively, RSM is a powerful technique that explores the relationships and interactions among multiple variables by reducing experimental trails. Until now, RSM has been successfully

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Table 1
Box–Behnken design matrix (in coded level of four variables) and response values for the yield of LBP.

Number	X_1 [a]	X_2 [b]	X_3 [c]	X_4 [d]	Polysaccharide yield (%)
1	−1 (10)	−1 (60)	0 (2.0%)	0 (60)	4.63
2	−1	1 (100)	0	0	4.47
3	1 (30)	−1	0	0	4.92
4	1	1	0	0	4.54
5	0 (20)	0 (80)	−1 (1.5%)	−1 (50)	4.72
6	0	0	−1	1 (70)	5.60
7	0	0	1 (2.5%)	−1	6.14
8	0	0	1	1	4.66
9	−1	0	0	−1	5.20
10	−1	0	0	1	4.92
11	1	0	0	−1	5.26
12	1	0	0	1	5.00
13	0	−1	−1	0	4.96
14	0	−1	1	0	5.29
15	0	1	−1	0	4.79
16	0	1	1	0	4.74
17	−1	0	−1	0	4.47
18	−1	0	1	0	4.96
19	1	0	−1	0	4.68
20	1	0	1	0	4.99
21	0	−1	0	−1	5.21
22	0	−1	0	1	5.43
23	0	1	0	−1	5.30
24	0	1	0	1	4.79
25	0	0	0	0	6.27
26	0	0	0	0	6.24
27	0	0	0	0	6.30

[a] Extraction time (min).
[b] Ultrasonic output power (W).
[c] Cellulose concentration (wt.% of *L. barbarum* powder).
[d] Temperature (°C).

applied in the optimization of conditions in food and pharmaceutical research frequently (Baş & Boyacı, 2007; Ghasemlou, Khodaiyan, Jahanbin, Gharibzahedi, & Taheri, 2012).

In the current study, the ultrasound-assisted enzymatic extraction (UAEE) of LBP was firstly investigated. A three-level four-factor BBD was employed combining with RSM to optimize the extraction conditions for obtaining the maximum yield of LBP.

2. Materials and methods

2.1. Materials and chemicals

Fruits of *L. barbarum*, family Solanaceae, were purchased from an herb market in Tianjin, China. Samples were ground and passed through 100 mesh screen.

Cellulose from *Trichoderma viride* (11000 U/mg) was supplied by Sigma Chemical Company. D-glucose was obtained from Tianjin Zhong Ao Tian Yuan Company, and the other chemicals were purchased from Tianjin Qian Cheng Wei Ye Company. All chemicals were reagent grade or better.

2.2. Methods

2.2.1. Instrument

The UAEE was performed in an ultrasound cleaning bath (KQ2200DB, Kunshan ultrasound instrument Co. Ltd., Jiangsu Province, China) working at a frequency of 40 kHz and a ultrasound input power of 40–120 W with a usable capacity of 3 L.

2.2.2. UAEE procedure

The powder of *L. barbarum* (20 g) was double extracted with petroleum ether at 90 °C for 3 h each time to remove lipids, some colored materials, and oligosaccharides under reflux in the Soxhlet set. After being vacuum dried at 60 °C for 12 h, the defatted powder (1 g) was mixed with 30 mL of cellulose solution at the given concentration and fixed pH 4.6 in a 100 ml conical flask (Zhang, Jia, Liu, Wu, & Ran, 2011). This mixture was ultrasonicated at the designed temperature, ultrasonic output power and extraction time. The extracting solution was concentrated, and then precipitated by adding ethanol (12 h, 4 °C) to a final concentration of 80% (v/v). The precipitate was collected and dried to obtain crude polysaccharides. The polysaccharides content was measured by phenol-sulfuric acid method using D-glucose as a standard (Dubios, Gilles, Hamilion, Rebers, & Smith, 1956). The percentage of LBP extraction yield (%) was calculated with the formula of $y\,(\%) = c/w \times 100\%$, where c was the content of polysaccharides, and w represented dried sample weight.

2.3. Box–Behnken design and statistical analysis

On the basis of single-factor experiments, RSM was used to further optimize the ultrasound-assisted enzymatic extraction conditions of LBP. A BBD with four independent variables (X_1, extraction time; X_2, ultrasonic output power; X_3, cellulose concentration; X_4, temperature) at three levels was performed. The range of independent variables and their levels of the independent variables and the results of whole design consisted of 27 experimental points carried out in random order were presented in Table 1, which were based on the results of preliminary experiments. For statistical calculation, each variable was coded at three levels: −1, 0, 1. The variables were coded according to the formula as follows:

$$x_i = \frac{X_i - X_0}{\Delta X}. \quad (1)$$

where x_i was a coded value of the variable; X_i was the actual value of variable; X_0 was the actual value of the X_i on the center point; and ΔX was the step change value.

From Table 1, three replicates (treatment 25–27) at the center of the design were used to allow for estimation the stability and variability (Ye & Huang, 2012). All trials were performed in triplicate.

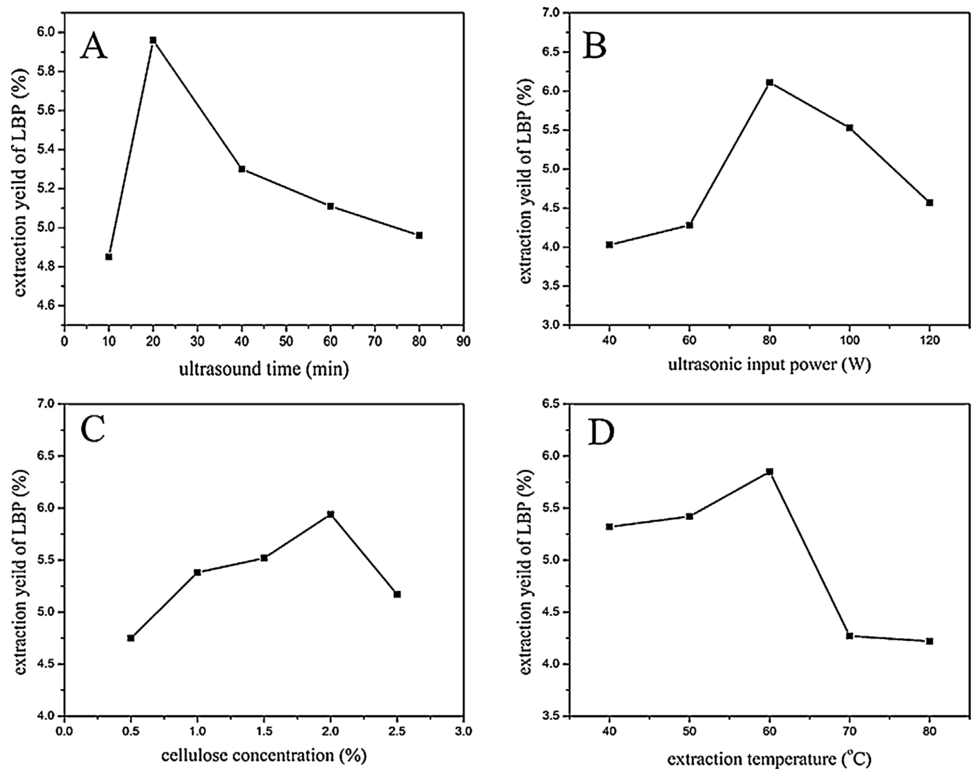

Fig. 1. Effect of different ultrasound time (A), ultrasound input power (B), cellulose concentration (C), and temperature (D) on the yield of LBP.

The extraction yield was treated as response. SAS (Version 9.2, USA) software package was used to generate the experimental design, statistical analysis and regression model.

Data from BBD were analyzed by multiple regressions to fit the following quadratic polynomial model.

$$y_k = b_{k0} + \sum_{i=1}^{4} b_{ki}x_i + \sum_{i=1}^{4} b_{kii}x_i^2 + \sum_{i<j=2}^{4} b_{kij}x_ix_j \quad (2)$$

y_k was the response function. b_{k0}, b_{ki}, b_{kii}, and b_{kij} represented the regression coefficients for intercept, linear, quadratic and interaction terms, respectively. And x_i and x_j were the independent variables. Additional confirmation experiments were subsequently conducted to verify the validity of the statistical experimental design.

3. Results and discussion

3.1. Effect of ultrasound time on the yield of polysaccharides

The effect of ultrasound time on extraction yield of polysaccharides was shown in Fig. 1(A). Extraction time was set at 10, 20, 40, 60, and 80 min to examine the influence on the yield of LBP when other extraction parameters were as follows: ultrasound input power of 100 W, cellulose concentration 2.0%, and temperature 60 °C. The yield of LBP increased with the increasing extraction time and reached the critical value 5.78% (±0.08%) when extraction time was 20 min, and then the curve began to decrease due to the loss of ultrasonicated post-processing (Chen et al., 2007).

3.2. Effect of ultrasound input power on the yield of polysaccharides

The yield of LBP by different ultrasound input power from 40 to 120 W was shown in Fig. 1(B), when other factors were as follows: ultrasound-assisted extraction time 20 min, cellulose concentration 2.0%, and extraction temperature 60 °C. The results indicated that the maximum yield of LBP (5.94 ± 0.12%) was reached when ultrasound input power was 80 W, and then it began to decrease, as chemical effect of ultrasonic is likely occurred under the powerful ultrasound (Chen et al., 2007).

3.3. Effect of cellulose concentration on the yield of polysaccharides

As shown in Fig. 1(C), cellulose was applied in the extraction process, and the effect of cellulose concentration on extraction yield was investigated. The enzyme concentrations were changed from 0.5% to 2.5%, while other extraction conditions were set as follows: ultrasound input power 80 W, ultrasound time 20 min and extraction temperature 60 °C. The curve of cellulose indicated that the yield of LBP increased with increase of cellulose concentration till 2.0% and then began to decrease, and the maximum extraction yield was 5.94% (±0.12%). So we adopted cellulose concentrations of 1.5–2.5% in the following experiment.

3.4. Effect of temperature on extraction yield of polysaccharides

The yield of LBP extracted by different temperature from 40 to 80 °C was shown in Fig. 1(D), when other factors were as follows: ultrasound input power 80 W, ultrasound time 20 min and cellulose concentration 2.0%. Results indicated that the maximum yield of LBP (5.94 ± 0.12%) was reached when extraction temperature was 60 °C, and then it began to decrease due to reduced enzyme activity at high temperatures (Fu et al., 2005).

3.5. Optimization of the procedure by RSM

3.5.1. Statistical analysis and the model fitting

There were a total of 27 runs for optimizing the four individual parameters in the BBD. The corresponding results and the design matrix of RSM experiments to determine the effects of the four independent variables including ultrasonic time (x_1); ultrasonic output power (x_2); cellulose concentration (x_3); temperature (x_4) were shown in Table 1. The mathematical model describing the extraction yield of LBP (y) as a function of the coded independent variables in the selected ranges was given by the following equation:

$$y = 6.27 + 0.061667x_1 - 0.150833x_2 + 0.13x_3 - 0.119167x_4 - 0.86625x_1^2 - 0.7375x_2^2 - 0.095x_2x_3 - 0.62125x_3^2 - 0.1825x_2x_4 - 0.59x_3x_4 - 0.3425x_4^2 \quad (3)$$

The coefficient values of Eq. (3) calculated and tested for their significance using SAS v 9.2 and the analysis results of fit statistics of extraction yield (y) for the selected quadratic predictive model were shown in Table 2.

The *P*-value was used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interactions between the variables. The smaller was the value of *P*, the more significant was the corresponding coefficient (Hou, 2008; Liu, Miao, Wen, & Sun, 2009). It can be seen from this table that the linear coefficients (x_1, x_2, x_3, x_4), quadratic term coefficients (${x_1}^2$, ${x_2}^2$, ${x_3}^2$, ${x_4}^2$) and cross product coefficients ($x_2 \times x_3$, $x_2 \times x_4$, $x_3 \times x_4$) were significant, with very small *P* values ($P < 0.05$). The other term coefficients were not significant ($P > 0.05$). The results indicated that the extraction time, ultrasonic output power, cellulose concentration and temperature were all significantly correlated with the LBP extraction yield.

Table 2
Test of significance for regression coefficients.

Effect	Estimate	Stand error	*t*-Ratio	*P*-value
x_1	0.061667	0.035658	10.93	<.0001[a]
x_2	−0.150833	0.020319	18.05	<.0001[a]
x_3	0.130000	0.812749	22.42	<.0001[a]
x_4	−0.119167	0.047797	14.79	<.0001[a]
$x_1 \times x_1$	−0.866250	0.000345	−25.14	<.0001[a]
$x_1 \times x_2$	−0.055000	0.000199	−1.38	0.1920
$x_2 \times x_2$	−0.737500	0.000086136	−21.41	<.0001[a]
$x_1 \times x_3$	−0.045000	0.007957	−1.13	0.2801
$x_2 \times x_3$	−0.095000	0.003978	−2.39	0.0343[a]
$x_3 \times x_3$	−0.621250	0.137818	−18.03	<.0001[a]
$x_1 \times x_4$	0.005000	0.000398	0.13	0.9021
$x_2 \times x_4$	−0.182500	0.000199	−4.59	0.0006[a]
$x_3 \times x_4$	−0.590000	0.007957	−14.83	<.0001[a]
$x_4 \times x_4$	−0.342500	0.000345	−9.94	<.0001[a]

$R^2 = 99.04\%$, Adj. $R^2 = 95.84\%$, CV = 1.55%. Lack of Fit: *F* value 8.24, Pr > *F* = 0.1130.
[a] Significant coefficient ($P < 0.05$).

The model has shown a good fit with the experimental data, as the value of coefficient of determination R^2 and adjusted determination coefficient Adj. R^2 were 99.04% and 95.84%, respectively. This confirmed that the fitted model could explain 99.04% of the total variability within the range of values studied, which was highly significant. What is more, the coefficient of the variation (C.V.) (1.55%) was not significant, which clearly indicated the precision and the experimental values of the model were of reliability.

The lack of fit is an indication of the failure for a model representing the experimental data at which points are not included in the regression or variations in the models cannot be accounted for random error (Pan et al., 2010). In our research, the lack of fit

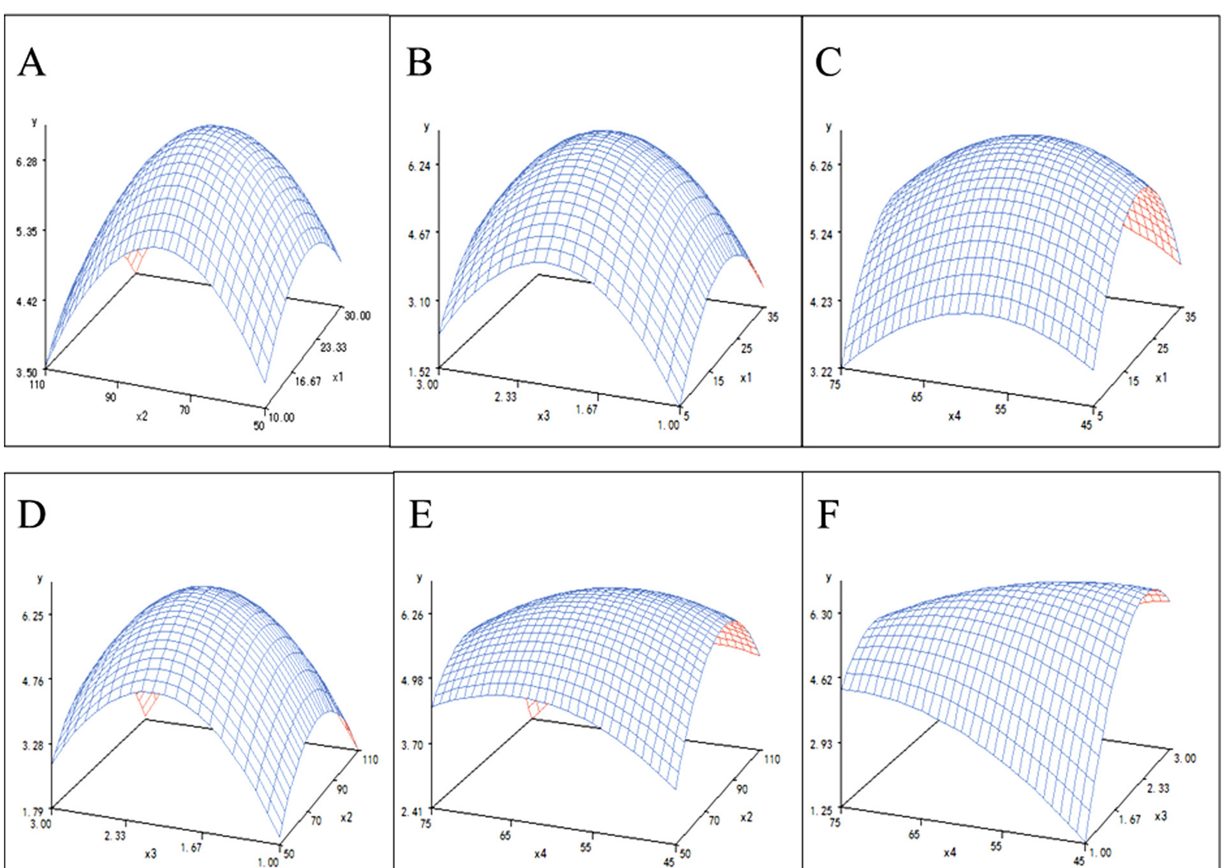

Fig. 2. The 3-D plots showing the effects of variables (X_1: ultrasonic time; X_2: ultrasonic output power; X_3: cellulose concentration; X_4: temperature) on extraction yield of LBP.

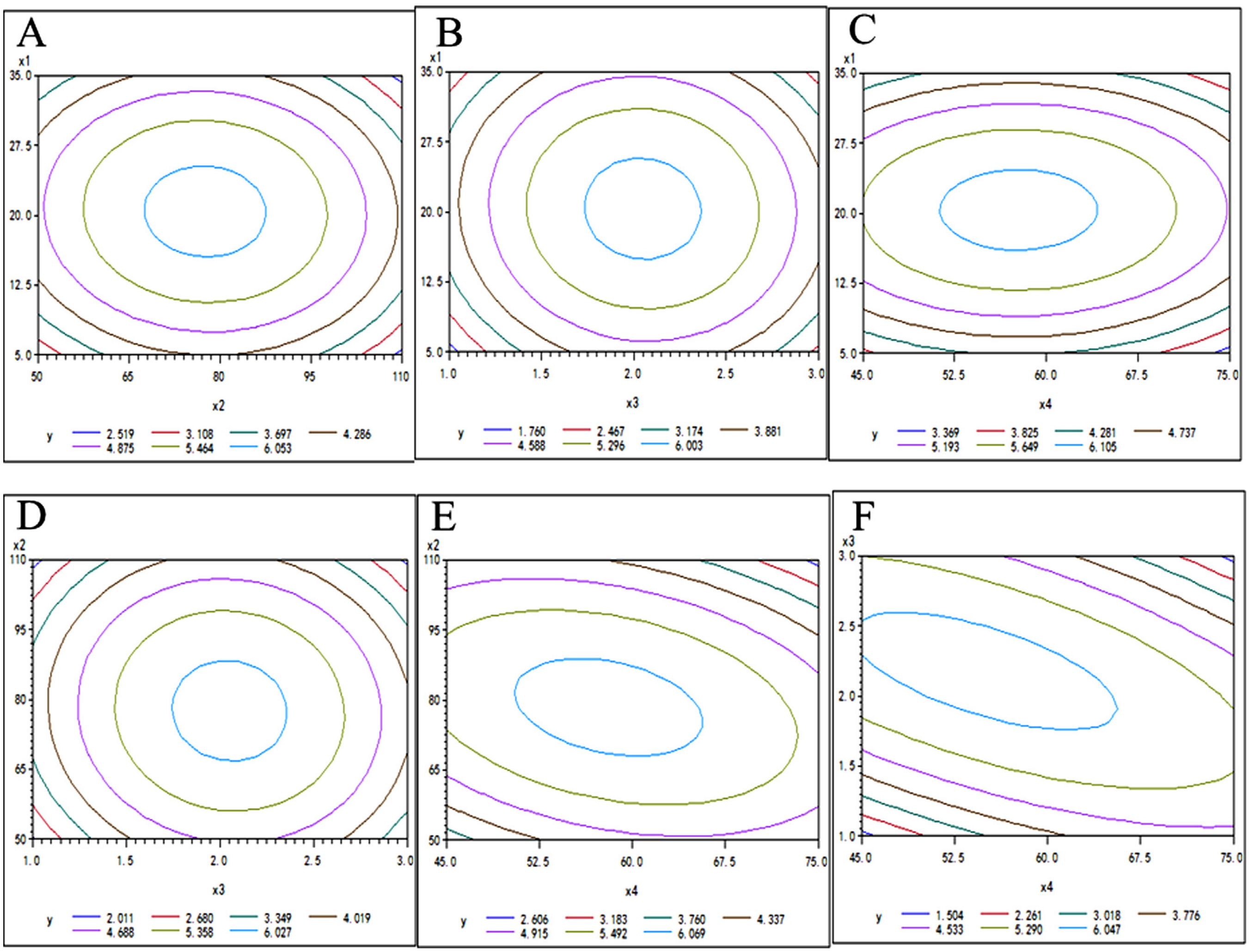

Fig. 3. The 2-D plots showing the effects of variables (X_1: ultrasonic time; X_2: ultrasonic output power; X_3: cellulose concentration; X_4: temperature) on extraction yield of LBP.

(0.1130) did not result in a significant *P*-value for selected variables in the model, which means that the model was sufficiently accurate for predicting the relevant response.

3.5.2. Response surface plots and contour plots showing effects of extraction variables on yield of polysaccharides

The graphical representations of the regression Eq. (3), called the response surfaces (3-D) presented in Fig. 2 and the contour plots (2-D) presented in Fig. 3 were obtained using SAS version 9.2. They provided a visual interpretation of interactions between two tested variables and the relationships between responses and experiment levels of each variable. Different shapes of the contour plots indicated different interactions between the variables. Elliptical contours were obtained when there was a perfect interaction between the independent variables while circular contour plots indicated otherwise (Guo, Zou, & Sun, 2010; Muralidhar, Marchant, & Nigam, 2001).

As shown in Figs. 2(A) and 3(A), where the extraction yield of LBP was given as a function of extraction time and ultrasonic output power at fixed cellulose concentration and temperature (0 level). The extraction yield of LBP increased with increase of extraction time from 10 to 20.29 min. But beyond 20.29 min, the extraction yield of LBP decreased with increasing extraction time when ultrasonic output power was set. Similarly, when the extraction time was set, the extraction yield of LBP was also found to increase rapidly with increase of ultrasonic output power from 50 to 78.6 W, and then there was a violent decrease when the extraction time continued to increase. And at this fixed cellulose concentration and temperature (0 level), the maximal LBP yield was 6.28%. The 2-D contour plot shown in Fig. 3(A) indicated that the mutual interaction between extraction time and ultrasonic output power was not significant.

Figs. 2(B) and 3(B) showed the 3-D response surface plot and the 2-D contour plot at varying extraction time and cellulose concentration at fixed ultrasonic output power and temperature (0 level). Figs. 2(B) and 3(B) indicated that extraction yield of LBP increased when the extraction time extended from10 to 20.29, and then began to decrease. At a fixed extraction time, the variety of extraction yield of LBP changed with cellulose concentration similarly as extraction time. The 2-D contour plot shown in Fig. 3(B) confirmed that the two variables of extraction time and cellulose concentration were relatively independent.

In Figs. 2(C) and 3(C), when the 3-D response surface plot and the contour plot were developed for the extraction yield of LBP with varying extraction time and extraction temperature at fixed ultrasonic output power and cellulose concentration (0 level), maximum extraction yield of LBP (6.28%) was achieved when extraction time and extraction temperature were 20.29 min and

Table 3
The comparison of different extraction processes of LBP.

Extraction procedures	Extraction conditions						The yield of LBP (%)
	A	B	C	D	E	F	
Enzyme-assisted	1.0	2.0	–	91	59.7	5.0	6.81
UAEE	–	2.15	80	20	56	4.6	6.31
Hot-water	–	–	–	180	80	7.0	3.88

A: papain concentration, B: cellulose concentration, C: ultrasonic output power, D: extraction time, E: extraction temperature, F: pH.

55.79 °C, respectively. The 2-D contour plot shown in Fig. 3(C) indicated that the mutual interaction between extraction time and extraction temperature was not significant ($P = 0.9021 > 0.05$).

Figs. 2(D) and 3(D) indicated the relationship between the yield of LBP and two variables of ultrasonic output power and cellulose concentration when extraction time and extraction temperature was set as 0 level. For Figs. 2(D) and 3(D) concerned, the extraction yield of LBP increased with increase of ultrasonic output power from 50 to 78.6 W, but beyond 78.6 W, extraction yield of LBP decreased with increasing ultrasonic output power when cellulose concentration was set. When ultrasonic output power was set, it indicated that extraction yield of LBP increased rapidly when cellulose concentration increased from 1% to 2.15%, and then began to decrease rapidly. It could be clearly shown that maximum extraction yield of 6.28% could be reached when extraction time and extraction temperature was set as 0 level. What shown in Fig. 3(D) was that there was certain mutual interaction ultrasonic output and cellulose concentration, which matched with the value of $P = 0.0343 < 0.05$.

What Figs. 2(E) and 3(E) showed was that the influence of change of ultrasonic output power and extraction temperature on the extraction yield of LBP when extraction time and cellulose concentration was set as 0 level. In the range of ultrasonic output power and extraction temperature, there was a maximum of the extraction yield of LBP. The 2-D contour plot shown in Fig. 3(E) indicated that the mutual interaction between ultrasonic output power and extraction temperature was significant ($P = 0.0006 << 0.05$). When extraction time was set as a constant, the extraction yield of LBP increased with increase of ultrasonic output power from 50 to 78.6 W, but beyond 78.6 W, extraction yield of LBP decreased with increasing ultrasonic output power. What is more, when extraction temperature was lower, the maximum of extraction yield of LBP was reached at a higher ultrasonic output power while a higher temperature indicated otherwise. On the other hand, the extraction yield of LBP varied with the change of extraction temperature presents a similar rule.

Figs. 2(F) and 3(F) indicated that the maximum (6.29%) of extraction yield of LBP obtained when extraction time and cellulose concentration was set as 0 level. Figs. 2(F) and 3(F) showed the influence of change of cellulose concentration and extraction temperature on the extraction yield of LBP when extraction time and ultrasonic output power was set (0 level). In the range of cellulose concentration and extraction temperature, when extraction temperature was lower, the extraction yield of LBP increased rapidly with the increase of cellulose concentration, and then the extraction yield of LBP decreased slightly with a continuous increase of cellulose concentration. Otherwise, when extraction temperature was higher, the extraction yield of LBP increased slightly with the increase of cellulose concentration, and then the extraction yield of LBP decreased rapidly with a continuous increase of cellulose concentration. When cellulose concentration was lower, the extraction yield of LBP increased with the increase of extraction temperature while a higher cellulose concentration indicated an opposite discipline. The 2-D contour plot shown in Fig. 3(F) indicated that the mutual interaction between cellulose concentration and extraction temperature was quite significant ($P < 0.0001 << 0.05$). From Figs. 2(F) and 3(F), a maximum of the extraction yield of LBP (6.32%) could be obtained when extraction time and ultrasonic output power was set (0 level).

According to Figs. 2 and 3, the optimal ultrasound-assisted enzymatic extraction conditions of LBP were as follows: extraction time 20.29 min, ultrasonic output power 78.6 W, cellulose concentration 2.15%, extraction temperature 55.79 °C. And the model predicted a maximum response of 6.32%. According to the regression coefficients significance of the quadratic polynomial model (Table 2) and gradient of slope in the 3-D response surface plot (Fig. 3), all the four extraction parameters studied were quite significant.

3.5.3. Verification of predictive model

The suitability of the model equation for predicting the optimum response values was tested using the selected optimal conditions. Because the optimal values of extraction time, ultrasonic output power, cellulose concentration, extraction temperature were difficult to operate, in the actual extraction experiments, they were carried out with slight modifications: extraction time 20 min, ultrasonic output power 80 W, cellulose concentration 2.15%, extraction temperature 56 °C, respectively. Under these conditions, the experiment yield of LBP was 6.31% (±0.03%), which was not significantly different ($P > 0.05$) from the predicted value of 6.32%. This proved that the model designed in this study was valid.

3.5.4. Comparison with other extraction

The optimum extraction process of UAEE in our study had some advantages when compared with other extraction methods. The results of comparison with other extraction were listed in Table 3. From Table 3, the yield of LBP obtained by UAEE under the optimum conditions in this study was 6.31% (±0.03%), which was almost similar to the yield of LBP of 6.81% obtained by enzyme-assisted extraction but much effective than it. Obviously, the extraction time of UAEE was just 20 min, which shortened extraction time greatly when compared to enzyme-assisted extraction whose extraction time was 91 min (Zhang et al., 2011). In addition, the yield of LBP obtained by UAEE was much higher and the extraction rate of UAEE was much faster than traditional hot-water treatment, which obtained a yield of 3.88% in the condition of extraction time 180 min and temperature 80 °C.

4. Conclusion

Ultrasound-assisted enzymatic extraction, which was an efficient and time-saving extraction technique, could be used to improve the extraction yield of LBP. Firstly, we acquired the optimal ranges of four main factors that affected the extraction yield of LBP significantly by the fundamental single-factor experiments. Then to further optimize LBP extraction conditions, extraction time, ultrasonic output power, cellulose concentration and extraction temperature were studied by RSM. The second-order polynomial model gave a satisfactory description of the experimental data. And the optimum point of extraction time 20 min, ultrasonic output power 80 W, cellulose concentration 2.15%, extraction temperature 56 °C was achieved vividly. Under the optimal conditions, the experiment extraction yield of LBP was 6.31% ± 0.03%, which was in accord with the predicted value of 6.32% closely.

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