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Extraction optimized by using response surface methodology, purification and preliminary characterization of polysaccharides from *Hyriopsis cumingii*

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ABSTRACT

Extraction, purification and preliminary characterization of polysaccharides from *Hyriopsis cumingii* (HCPS) were carried out. Firstly, the extracting parameters for HCPS extraction were optimized by using three-factor-three-level Box–Behnken design and response surface methodology based on the single-factor experiments. As results, the optimum conditions were extracting temperature 80 °C, extracting time 4.5 h and the ratio of extraction solvent (water) to raw material (v/w) 8. Under these conditions, the experimental yield of crude HCPS was 3.66%. Secondly, the crude HCPS was purified by DEAE-Cellulose 52 chromatography and Sephadex G-100 chromatography to afford 3 fractions, HCPS-1, HCPS-2 and HCPS-3. The recovery rates based on crude HCPS used were 26.91%, 30.18% and 3.93% for HCPS-1, HCPS-2 and HCPS-3 respectively. Based on the characterization results of HCPS by Fourier transform-infrared spectroscopy, high performance liquid chromatography and gas chromatography, we found that HCPS-3 was quite different from HCPS-1 or HCPS-2.

1. Introduction

Polysaccharide-containing extracts from plants, epiphytes and animals have been widely used for the treatment of some diseases in traditional Chinese medicine. Mussel is one of the interesting bioactive polysaccharide resources. It has been reported that the polysaccharides from mussel have potential biological functions, for instance anti-tumor, anti-virus, immunity-modulation, anti-oxidation and so on (Lei, Zhong, Yang, & Zhu, 2006; Miller, Dodd, Ormrod, & Geddes, 1993; Xu et al., 2008; Zhang, Wu, Di, & Chen 2007). Therefore, discovery and evaluation of new polysaccharides from the various mussels has become a hot research spot.

Hyriopsis cumingii, a member of freshwater pearl mussels, is the most economically important mussel species in China. In the world market, China produces 95% of freshwater pearls and 95% of freshwater pearls are produced from *H. cumingii* (Cheng & Li, 2007; Hua & Gu, 2002). As the best for producing high quality pearls, *H. cumingii* is widely cultivated in China. However, the *H. cumingii* flesh (waste of producing freshwater pearls) is used mainly as animal feeds and fertilizer. In fact, its flesh can be used as the materials of food and medicine. It has been reported that *H. cumingii* can cure some diseases in traditional Chinese medicine. In addition, recent studies demonstrate that the *H. cumingii* flesh is rich in polysaccharides, which resulting in nutrient and pharmacological functions such as anti-tumor, immunity-enhancement, anti-inflammation and anti-aging (Cheng, Wu, Lu, & Chi, 2007; Hu & Cao, 2003; Zhang et al. 2007). However, little attention has been devoted to the extraction, purification, characterization and biological functions of *H. cumingii* polysaccharides (HCPS). Herein, we report in detail the optimization of extracting parameters for the production of HCPS, the purification and the preliminary characterization of HCPS prepared.

The response surface methodology (RSM) has been extensively utilized to optimize culture conditions and medium composition of fermentation process, conditions of enzyme reaction, and processing parameters in the production of food and drug (Hou & Chen, 2008; Kshirsagar & Singhal, 2007; Masmoudi et al., 2008; Song, Zhang, Kuang, Zhu, & Guo, 2007). Box–Behnken design (BBD), one of RSM, only have three-levels (low, medium and high, coded as −1, 0 and +1), and need fewer experiments. It is more efficient and easier to arrange and interpret experiments in comparison with others (Box & Behnken, 1960; Ferreira et al., 2007; Wang & Lu, 2005). Therefore, BBD of RSM was used to optimize the extracting parameters of HCPS in the present work. Firstly, single-factor experimental designs (extracting temperature, extracting time, extracting times, and ratio of extracting solvent to raw material) were carried out before RSM experiments. Secondly, three factors (extracting temperature, extracting time and ratio of water to raw material) were chosen based on single-factor designs for further optimization by employing a three-level, three-variable BBD from RSM. Furthermore, HCPS was purified through anion-ex-

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change chromatography and size-exclusion chromatography and the isolated fractions were characterized using Fourier transform-infrared spectroscopy (FT-IR), high performance liquid chromatography (HPLC) and gas chromatography (GC).

2. Materials and methods

2.1. Materials and reagents

All mussel *H.* cumingii were collected from Jiangning Pearl Farm (Nanjing, China). Arabinose, rhamnose, fucose, xylose, galactose, glucose, mannose, glucan, DEAE-cellulose 52 and Sephadex G-100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Extraction of polysaccharides

The extraction of HCPS was done according to the reported method with some modifications (Yao et al., 2005). Briefly, fresh *H. cumingii* were collected and cleaned carefully with water. After removing the shells, the flesh was preserved in 75% ethanol for use. Preserved *H. cumingii* flesh was ground in a high speed disintegrator. The homogenates were diluted with deionized water (ratio of water to raw material, 5 to 20 in v/w) and incubated in thermostat-controlled water-bath (60 to 90 °C) for 2 to 5 h. After incubation, the mixtures were centrifuged at 5000 r/min for 15 min, and the insoluble residue was treated again for 2–3 times as mentioned above. The supernatants were collected and concentrated by a rotary evaporator to a proper volume. The resulting solution was mixed with three volumes of dehydrated ethanol (ethanol final concentration, 75%) and kept for 24 h at room temperature. Then the solution was centrifuged at 5000 r/min for 15 min, and the precipitate from ethanol dispersion was collected as crude extract. The crude extract obtained was dissolved in deionized water, deproteinated by the method of Sevag (Sevag, Lackman, & Smolens, 1938) for 10 times and concentrated by rotary evaporator and mixed with dehydrated ethanol to a final ethanol concentration of 90%. Finally, the precipitate from centrifugation (5000 r/m, 15 min) was dissolved in deionized water and lyophilized to afford crude HCPS.

2.3. Experimental design of RSM

On the basis of single-factor experiment for the polysaccharides production, proper ranges of extraction time, extraction temperature, ratio of water to raw material and extraction times were preliminarily determined. A three-level, three-variable BBD (software Design-Expert v.6.0.10, Stat-Ease, Inc, Minneapolis, USA) was applied to determine the best combination of extraction variables for the production of polysaccharides. Based on the investigations on single-factor experiment, the variables considered are extraction time, extraction temperature and ratio of water to raw material in this experimental design. Table 1 lists BBD matrix and the response values that were carried out for developing the model. The whole design consisted of 17 experimental points carried out in random order. Five replicates (treatment 13–17) at the centre of the design were used to allow for estimation of a pure error sum of squares. The response value in each trial was average of duplicates.

Based on the experimental data, regression analysis was performed and was fitted into an empirical second-order polynomial model:

$$Y = \sum A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} X_i X_j$$

where Y is the response variable, A_0, A_i, A_{ii}, A_{ij} are the regression coefficients of variables for intercept, linear, quadratic and interaction terms respectively, and X_i, X_j are independent variables ($i \neq j$). The coefficients of the second polynomial model and the responses obtained from each set of experimental design were subjected to multiple nonlinear regressions using software Design-Expert. The fitness of the polynomial model equation was expressed by the coefficient of determination R^2, and its statistical significance was checked by F-test at a probability (P) of 0.001, 0.01 or 0.05. The significances of the regression coefficients were also tested by F-test.

2.4. Purification of crude polysaccharides

The crude HCPS was separated and sequentially purified through a column of DEAE-cellulose 52 and a column of Sephadex G-100 according reported methods with slight modification (Bendahou, Dufresne, Kaddami, & Habibi, 2007; Ye, Wang, Zhou, Liu, & Zeng, 2008). Crude HCPS solution (15 mg/ml, 3 ml) was applied to DEAE-Cellulose 52 column (2.6 × 30 cm). Then, the column was stepwise eluted with 0.0, 0.1, 0.3, 0.5 and 0.7 M sodium chloride solutions at a flow rate of 60 ml/h. Eluate (10 ml/tube) was collected automatically and the carbohydrates were determined by the phenol–sulfuric acid method. As results, three fractions of polysaccharides were obtained, concentrated, dialyzed and further purified through Sephadex G-100 column (2.6 × 60 cm) to afford HCPS-1, HCPS-2 and HCPS-3, respectively. Finally, purified HCPS-1, HCPS-2 and HCPS-3 were lyophilized for further study.

2.5. Analysis of polysaccharides characterization

FT-IR of polysaccharides was carried out by the potassium bromide (KBr) pellet method on Fourier transform-infrared spectrometer type MB154S (Bomen, Canada) in the range of 500–4000 cm^{-1}. The contents of carbohydrate, protein, uronic acid and sulfate in HCPS were determined according to the reported methods respectively (Bradford, 1976; Dodgson & Price, 1962; Dubois, Gilles, Hamilton, Rebers, & Smith, 1956; Karamanos, Hjerpe, Tsegenidis, Engfeldt, & Antonopoulos, 1988). Relative viscosity (to deionized water) of HCPS was measured in Ubbelohde Viscometer (Dazhan Institute of Machine and Electronics, Nanjing, China) at a concentration of 10 mg/ml and 25 °C. The analysis of monosaccharide compositions of HCPS was carried out by GC (GC-6890N, Agilent) with a flame ionization detector and a HP-5 capillary column (30 m × 0.32 mm × 0.25 μm). The operation conditions of GC were as following: flow rates of N_2, H_2 and air were 25, 30 and 400 ml/min, respectively; the temperatures of oven, detector and inlet were 210, 280 and 250 °C, respectively. The relative molecular weight of HCPS was determined by HPLC (1100 Series HPLC, Agilent) equipped with a refractive index detector and a TSK-Gel G3000SW_{XL} column (7.8 × 300 mm) (Tosoh Corp., Tokyo) eluted with 0.1 M Na_2SO_4 at a flow rate of 0.8 ml/min at 25 °C. The column was calibrated with standard glucans and a standard curve was then established.

3. Results and discussion

3.1. Effect of extracting temperature on the yield of HCPS

To investigate the effect of extracting temperature on the yield of HCPS, extraction process was carried out using different extraction temperature of 60, 70, 80 and 90 °C, while other extracting parameters were fitted as following: extracting time 4 h, extracting ratio of water to raw material 20 and extracting number 3 times. As shown in Fig. 1A, there was an increasing trend in the yield of HCPS from 60 to 90 °C. This tendency was in agreement with other

Table 1
Box–Behnken design matrix and the response values for the yield of HCPS.

Standard order	Temperature(x_1/°C)	Time (x_2/h)	Ratio of water to raw material (x_3)	Polysaccharide yield (%)	
				Experimental	Predicted
1	70	3	10	1.5052	1.4846
2	90	3	10	2.1245	2.2173
3	70	5	10	2.8874	2.7947
4	90	5	10	2.6924	2.7130
5	70	4	5	3.2587	3.3223
6	90	4	5	2.6149	2.5651
7	70	4	15	1.2911	1.3409
8	90	4	15	2.8127	2.7491
9	80	3	5	2.1839	2.1409
10	80	5	5	3.3989	3.4281
11	80	3	15	1.6556	1.6264
12	80	5	15	2.1022	2.1452
13	80	4	10	3.5629	3.5397
14	80	4	10	3.5986	3.5397
15	80	4	10	3.6283	3.5397
16	80	4	10	3.4961	3.5397
17	80	4	10	3.4126	3.5397

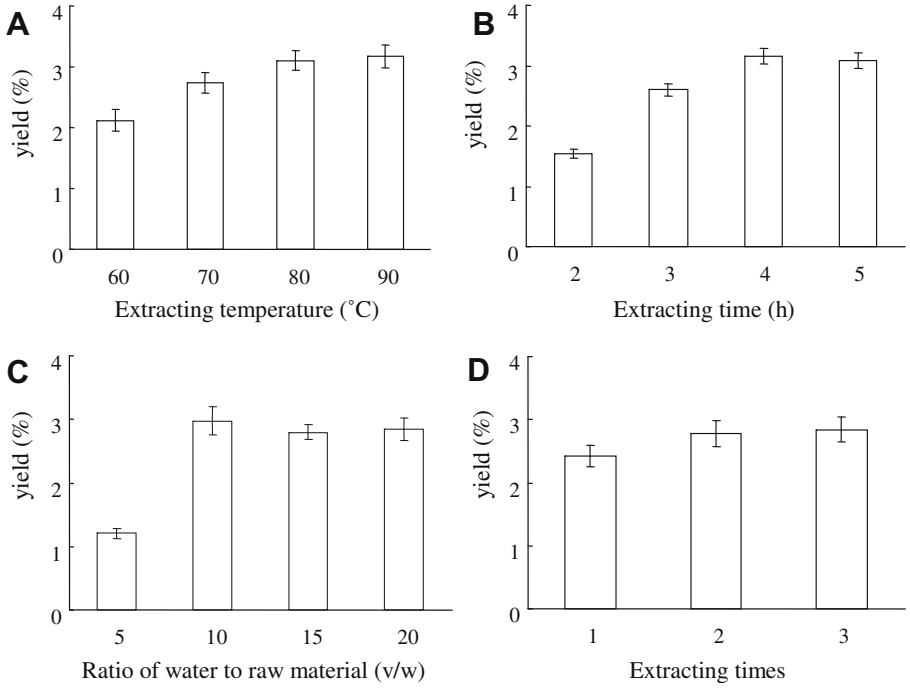

Fig. 1. Effects of extracting temperature (A), extracting time (B), ratio of water to raw material (C) and extracting times (D) on the yield of HCPS.

reports in extracting polysaccharides (Vinogradov, Brade, Brade, & Holst, 2003). Statistical analysis showed that significant differences were existing among 60 °C, 70 °C and 80 °C, and among 60 °C, 70 °C and 90 °C ($P < 0.05$), but there was no significant difference between 80 °C and 90 °C ($P > 0.05$). These results indicate that there is a positively significant effect of extracting temperature on the yield of HCPS when less than 80 °C, the effect is not significant when extracting temperature is higher than 80 °C. Therefore, 80 °C was selected as the centre point of extracting temperature in the RSM experiments as higher temperature will bring about the energy waste and cost increase for extraction process.

3.2. Effect of extracting time on the yield of HCPS

Extraction time was another factor that would influence the extraction efficiency and selectivity of the fluid. It has been reported that a long extraction time favored the production of polysaccharides (Liu, Wei, Guo, & Kennedy, 2006). On the other hand, excessive lengthening of extraction time may induce the change of polysaccharides molecule structure (Cai, Gu, & Tang, 2008). In the present study, the effect of extracting time on the yield of HCPS was investigated using different extracting time (2, 3, 4 and 5 h) while other extraction variables were set as follows: extracting temperature 80 °C, extracting ratio of water to raw material 20 and extracting times 3. The results showed that there was an increasing trend in the yield of HCPS with the increasing of extracting time in the range of 2–4 h (Fig. 1B). Statistical analysis showed that significant differences were existent among 2 h, 3 h and 4 h, and among 2 h, 3 h and 5 h ($P < 0.05$), but there was no significant difference between 4 h and 5 h ($P > 0.05$). Thus, extraction time of 4–5 h is favorable for the production of HCPS. For saving of energy and lowering of cost, 4 h was selected as the centre point of extracting time in the RSM experiments.

3.3. Effect of extracting ratio of water to raw material on the yield of HCPS

Ratios of water to raw material were set at 5, 10, 15 and 20 in order to investigate the effect of different extracting ratio of water to raw material on the yield of HCPS. In the range of 5–20 for the ration of water to raw material, significant differences were existent between 5 and 10, 5 and 15, 5 and 20 ($P < 0.05$), but there were no significant differences among 10, 15 and 20 ($P > 0.05$) as shown in Fig. 1C. The yields of HCPS significantly increased from 1.20 to 2.98% as the ratio of water to raw material increased from 5 to 10. This may due to the increase of the driving force for the mass transfer of the polysaccharides (Bendahou et al., 2007). Therefore, 10 were selected as the centre point of extracting ratio of water to raw material in the RSM experiments.

3.4. Effect of extracting times on the yield of HCPS

Fig. 1D showed the effect of extraction times on the yield of HCPS while other extracting parameters were fitted as follows: extracting temperature 80 °C, extracting time 4 h, and extracting ratio of water to raw material 20. From Fig. 1D, we found that there was an increasing trend in the yield of HCPS accompanying the increase of extracting times, but there was not significant difference ($P > 0.05$) between 2 times and 3 times. Taking the yield and processing cost into consideration, 2 times was sufficient for the extraction of polysaccharides. Thus, 2 times was selected as the extracting times in the next experiments.

3.5. Predicted model and statistical analysis

The values of responses (yield of HCPS) at different experimental combination were given in Table 1. It can be seen from Table 1 that there was a considerable variation in the yield of HCPS depending upon the extraction conditions. The application of RSM offered, based on parameter estimates, an empirical relationship between the response variable and the test variables. By employing multiple regression analysis on the experimental data, the predicted response Y for the yield of polysaccharides can be obtained by the following second-order polynomial equation: $Y = 3.5397 + 0.1628x_1 + 0.4515x_2 - 0.4494x_3 - 0.5391x_1^2 - 0.6983x_2^2 - 0.5063x_3^2 - 0.2036x_1x_2 + 0.5414x_1x_3 - 0.1921x_2x_3$, where x_1, x_2 and x_3 were the coded values of the test variables, extracting temperature (°C), extracting time (h) and ratio of water to raw material, respectively.

The statistical significance of regression equation was checked by F-test, and the analysis of variance (ANOVA) for response surface quadratic polynomial model was done by software Design-Expert. The ANOVA of quadratic regression model demonstrated that the model was highly significant. And the Fisher's F-test had a very high model F-value (114.74) and a very low P-value ($P < 0.0001$). The goodness of the model can be checked by the determination coefficients (R^2) and the multiple correlation coefficients (R). Closer the values of R to 1, better the correlation between experimental and predicted values (Pujari & Chandra, 2000). In this experiment, the value of R (0.9966) indicates good agreement between the experimental and predicted values of the yield of HCPS. The value of adj-R^2 (0.9846) suggests that the total variation of 98% for the yield of HCPS was attributed to the independent variables and only about 2% of the total variation cannot be explained by the model.

The lack-of-fit measures the failure of the model to represent the data in the experimental domain at points which are not included in the regression. The F-value (1.63) and P-value (0.3174) of lack-of-fit implied the lack-of-fit was not significant relative to the pure error. It indicates that the model equation is adequate for predicting the yield of HCPS under any combination of values of the variables.

The coefficient estimates of model equation, along with the corresponding P-values, were presented in Table 2. The P-values are used as a tool to check the significance of each coefficient, which also indicate the interaction strength between each independent variable. Smaller the P-value is, more significant the corresponding coefficient is (Muralidhar, Chirumamila, Marchant, & Nigam, 2001). When value of "probability > F" is less than 0.05, the model terms is significant. It can be seen from Table 2 that all regression coefficients were highly significant, and x_1, x_2, x_3, x_1^2, x_2^2, x_3^2, x_1x_2, x_1x_3, x_2x_3 were significant model terms.

3.6. Response surface plot and contour plot

The 3D response surface and 2D contour plots are the graphical representations of regression equation. They provide a method to visualize the relationship between responses and experimental levels of each variable and the type of interactions between two test variables. The shapes of the contour plots, circular or elliptical, indicate whether the mutual interactions between the variables are significant or not. Circular contour plot indicates that the interactions between the corresponding variables are negligible, while elliptical contour plot indicates that the interactions between the corresponding variables are significant (Muralidhar et al., 2001). In the present study, three independent response surface plots and their respective contour plots were generated using Design-Expert as shown in Fig. 2. It is clear that the yield of HCPS is sensitive to minor alterations of the test variables (temperature, time and ratio of water to raw material). In addition, all the mutual interactions among the tested variables are significant.

Through these three-dimensional plots and their respective contour plots, it is very easy and convenient to understand the

Table 2
Regression coefficients estimate and their significance test for the quadratic polynomial model.

Model term	Coefficient estimate	Standard error	Sum of squares	Mean square	F-value	Probability (P) > F	Significance
Intercept	3.54	0.044					
x_1	0.16	0.034	0.21	0.21	22.34	0.0021	**[a]
x_2	0.45	0.034	1.63	1.63	171.88	<0.0001	***[b]
x_3	−0.45	0.034	1.62	1.62	170.28	<0.0001	***
$x_1 \times x_1$	−0.54	0.047	1.22	1.22	128.98	<0.0001	***
$x_2 \times x_2$	−0.70	0.047	2.05	2.05	216.41	<0.0001	***
$x_3 \times x_3$	−0.51	0.047	1.08	1.08	113.77	<0.0001	***
$x_1 \times x_2$	−0.20	0.049	0.17	0.17	17.47	0.0041	**
$x_1 \times x_3$	0.54	0.049	1.17	1.17	123.57	<0.0001	***
$x_2 \times x_3$	−0.19	0.049	0.15	0.15	15.56	0.0056	**

[a] Significance at 0.01 level.
[b] Significance at 0.001 level.

interactions between two variables and to locate their optimum ranges. By analyzing the plots (Fig. 2), the optimal values of the tested variables for obtaining HCPS of approximate 3.54% lie in the following ranges: extracting temperature 77.12–84.17 °C, extracting time 3.97–4.65 h, and ratio of water to raw material 5.00–10.16. From the above analysis, the best value of HCPS yield occurs at a low ratio of water to raw material (Fig. 2D and Fig. 2F), an appropriate extracting temperature (Fig. 2B and Fig. 2D) and a long extracting time (Fig. 2B and Fig. 2F).

3.7. Optimization of extracting parameters and validation of the model

By employing the software Design-Expert, the solved optimum values of the tested variables were extracting temperature

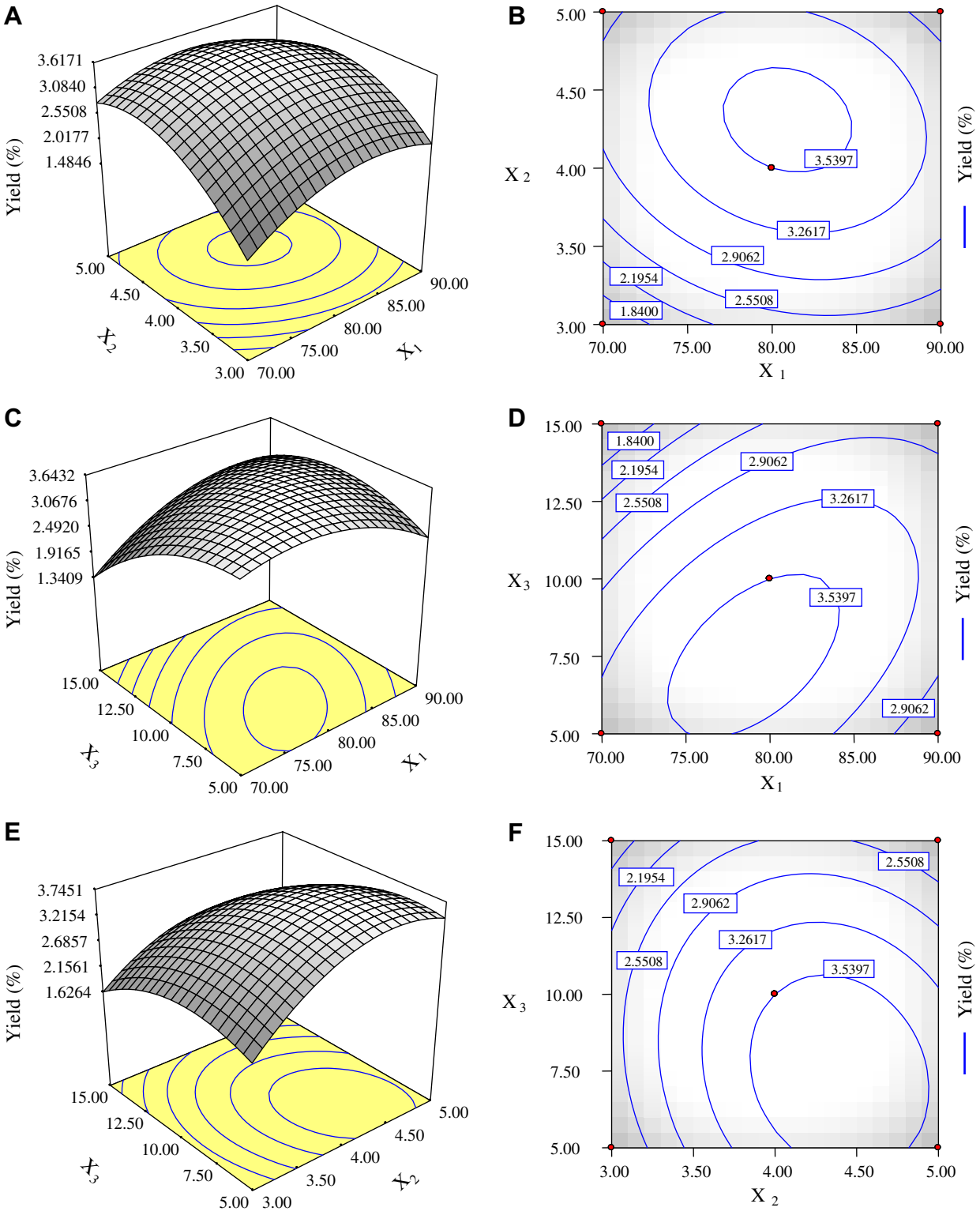

Fig. 2. Response surface plots (A, C and E) and contour plots (B, D and F) showing the effect of extracting temperature (X_1), extracting time (X_2) and ratio of water to raw material (X_3) on the yield of HCPS.

Table 3
Model validation experiments.

Trial order	Temperature (°C)	Time (h)	Ratio of water to raw material (v/w)	Polysaccharide yield (%)	
				Experimental	Predicted
1	75	3.5	8	3.1137	3.0409
2	75	4.5	10	3.3218	3.4256
3	80	3.5	6	3.1349	3.0980
4	80	4.0	6	3.4962	3.5752
5	80	4.0	10	3.5069	3.5397
6	80	4.5	8	3.6851	3.7280
7	80	4.5	8	3.6226	3.7280
8	80	4.5	8	3.7319	3.7280
9	80	4.5	8	3.5982	3.7280
10	80	4.5	8	3.6735	3.7280

79.85 °C, extracting time 4.46 h and ratio of water to raw material 8.08. Under the optimal conditions, the maximum predicted yield of HCPS was 3.74%, slightly higher than that obtained from the plot analysis. Taking account of the operating convenience, the optimal parameters were determined as following: temperature 80°C, time 4.5 h and the ratio of water to raw material 8. Using these parameters, the predicted yield of HCPS was about 3.73%, slightly less than that of the maximum predicted value.

In order to validate the adequacy of the model equation, five verification experiments were carried out under various extracting conditions (within the experimental range). The suitability of the optimal extracting variables was also tested by executing five experiments under these conditions: extracting temperature 80 °C, extracting time 4.5 h and ratio of water to raw material 8. Table 3 presents the design matrix along with the experimental results and theoretical values predicted by regression equation. The correlation coefficient (R) between the experimental and predicted values was 0.9823. There was not statistically difference at significant level of 0.05 between the experimental and predicted values. The results indicate that the experimental values are in good agreement with the predicted ones, and also suggest that the regression model is accurate and adequate for the extraction of HCPS.

3.8. Purification and characterization of HCPS

The crude HCPS solution was firstly separated through an anion-exchange chromatography column of DEAE-cellulose 52, it afforded three independent elution peaks (F_1, F_2 and F_3) as detected by the phenol-sulfuric acid assay (Fig. 3A). The 3 fractions were further loaded onto a column of Sephadex G-100 respectively, and the column was eluted with deioned water. As results, each fraction generated one single elution peak (Fig. 3B–D), named as HCPS-1, HCPS-2 and HCPS-3 respectively. The recovery rates of HCPS-1, HCPS-2 and HCPS-3 based on the amount of crude HCPS used were 26.91%, 30.18% and 3.93%, respectively.

The crude and purified HCPS were preliminary characterized by HPLC, GC and FT-IR. Table 4 shows the relative viscosities (to deionized water), relative molecular weights, contents of carbohydrate, protein, uronic acid and sulfate in crude HCPS, HCPS-1, HCPS-2 and HCPS-3. We found that HCPS-3 was quite different from the crude HCPS, HCPS-1 and HCPS-2. HCPS-3 contained 9.42% of protein, indicating that it may be proteoglycan. In addition, HCPS-3 had much higher sulfate content and relative higher relative viscosity.

The monosaccharide compositions of HCPS were analyzed. The crude HCPS was composed of rhamnose, arabinose, fucose, mannose, glucose and galactose with a molar ratio of 5.71:2.21:1.69:3.40:82.21:4.78. HCPS-1 was composed of only arabinose and glucose with a molar ratio of 2.12:97.88. HCPS-2 was composed only glucose. HCPS-3 was composed of rhamnose, fucose, mannose, glucose and galactose with a molar ratio of 13.80:4.51:7.70:64.92:9.07. These results indicate that monosaccharide composition of HCPS-3 was more complicated than that of HCPS-1 or HCPS-2.

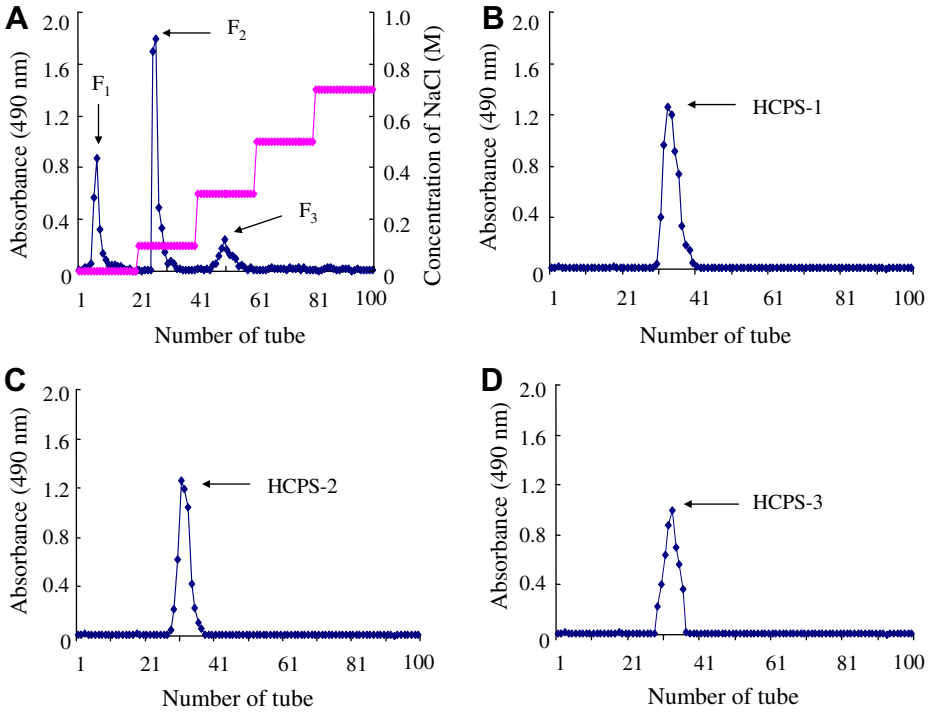

Fig. 3. Stepwise elution curve of crude HCPS on anion-exchange chromatography column DEAE-Cellulose 52 (A) and elution curve of polysaccharides fractions (F_1, F_2 and F_3) from DEAE-Cellulose 52 on size-exclusion chromatography column Sephadex G-100 (B–D).

Table 4
Preliminary characterization of crude HCPS, HCPS-1, HCPS-2 and HCPS-3.

Item	Crude HCPS	HCPS-1	HCPS-2	HCPS-3
Carbohydrate (%)	76.4250	98.8826	96.6001	80.0580
Protein (%)	1.3611	–[a]	–	9.4238
Uronic acid (%)	13.1663	16.6600	16.3732	17.2492
Sulfuric radical (%)	1.3803	0.3804	0.5959	6.2938
Relative viscosity (to water)	1.1810	1.1592	1.1631	1.3080
molecular weight (kDa)		432.2	457.9	503.1

[a] Not detected.

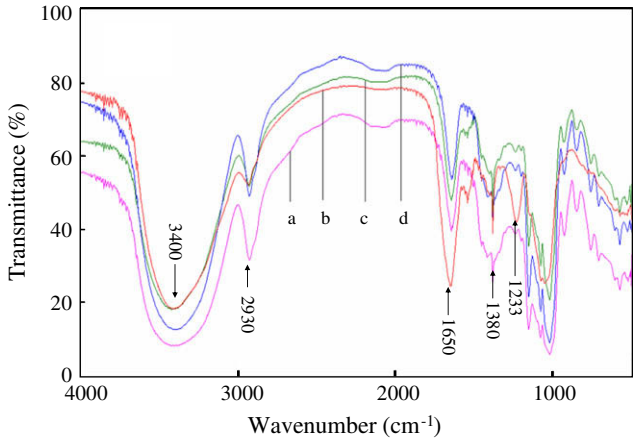

Fig. 4. FT-IR spectra of HCPS-1 (a), HCPS-3 (b), crude HCPS (c), and HCPS-2 (d).

The FT-IR spectra of crude HCPS, HCPS-1, HCPS-2 and HCPS-3 are showed in Fig. 4. Two characteristic absorptions of polysaccharides, a strong and wide absorption band of about 3100–3700 cm^{-1} for O–H stretching vibrations and a strong absorption peak of about 2800–3000 cm^{-1} for C–H stretching vibrations, were observed. Two characteristic absorptions, a peak of about 1600–1700 cm^{-1} (C=O asymmetric stretching vibrations) and a band of about 1300–1400 cm^{-1} (C=O symmetric stretching vibrations), indicated that there were carboxyl groups in HCPS. It could be verified by the fact that all crude and purified HCPS had relative higher uronic acid contents (Table 4). In addition, absorption peak of about 1600–1700 cm^{-1} was also for N–H bending vibration. And HCPS-3 showed the strongest absorption peak of about 1600–1700 cm^{-1} (Fig. 4b), it might be related to its high protein content (Table 4). Finally, an absorption peak of about 1230–1240 cm^{-1} (S=O stretching vibrations) in HCPS-3 was stronger than that in crude HCPS, HCPS-1 or HCPS-2. It means that HCPS-3 should have more sulfuric acid radicals than crude HCPS, HCPS-1 or HCPS-2. And the results of Table 4 were in coincidence with the results of FT-IR characterization.

4. Conclusion

Based on the single-factor experiments, BBD from RSM was used for optimizing extraction parameters in this work. Through optimization, the optimal conditions for the production of HCPS are as the following: extracting temperature 80 °C, extracting time 4.5 h and the ratio of water to raw material 8. Under these conditions, the experimental yield of crude HCPS was 3.66%. Three fractions of polysaccharides (HCPS-1, HCPS-2 and HCPS-3) were obtained through purification of the crude HCPS. The recovery rates of HCPS-1, HCPS-2 and HCPS-3 based on crude HCPS used were 26.91%, 30.18% and 3.93%, respectively. HCPS-3 was quite different from the crude HCPS, HCPS-1 and HCPS-2. As HCPS-3 contained 9.42% of protein, much higher sulfate content, relative higher relative viscosity and relative complicated monosaccharide composition, it should be investigated in detail in future works. The studies on the chemical structures and biological functions of HCPS such as anti-oxidation, antitumor, antibacterial and immunity-modulation are in progress.

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