Carbohydrate Polymers 74 (2008) 603–610

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Optimization of extraction technology of the *Lycium barbarum* polysaccharides by Box–Behnken statistical design

Guihao Yin [a,*], Yuli Dang [b]

[a] *Analysis and Test Center of Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, PR China*
[b] *College of sciences, Henan Agricultural University, Zhengzhou 450002, PR China*

ARTICLE INFO

Article history:
Received 6 February 2008
Received in revised form 2 April 2008
Accepted 10 April 2008
Available online 24 April 2008

Keywords:
Polysaccharides
Lycium barbarum
Optimization
Response surface methodology
Box–Behnken design

ABSTRACT

The quantitative effects of extraction time, extraction temperature, ratio of water to raw material and number of extraction on yield of *Lycium barbarum* polysaccharides were investigated using response surface methodology (RSM). The four factors chosen for the present investigation were based on the results of a single-factor test. The experimental data obtained were fitted to a second-order polynomial equation using multiple regression analysis and also analyzed by appropriate statistical methods. By solving the regression equation and also by analyzing the response surface contour plots, the optimal polysaccharides extraction conditions were determined: extraction time 5.5 h, extraction temperature 100 °C, ratio of water to raw material 31.2 and number of extraction 5. These predicted values were also verified by validation experiments. At last, four isolated fractions of the *L. barbarum* polysaccharides were characterized by employing GC.

1. Introduction

Lycium barbarum is a perennial bush that can be found in most areas of China and countries in Europe and around the Mediterranean. In China, it has been used for centuries in traditional herbal medicine as an important source of medicine and as the base for a valuable nourishing tonic (Committee of Chinese Pharmacopoeia 1990). Its red fruits contain 18 kinds of amino acids (six times higher than bee pollen), all 8 essential amino acids (such as isoleucine and tryptophan) and 21 trace minerals (the main ones being zinc, iron, copper, calcium, germanium, selenium, and phosphorus) (Cui, Xing, Liu, Xing, & Wang, 1996). The red fruits are the richest source of carotenoids, including beta-carotene (more beta-carotene than carrots), of all known foods or plants on earth. They contain 500 times the amount of vitamin C, by weight, than oranges making them second only to camu camu berries as the richest vitamin C source on earth. They also contain vitamins B1, B2, B6, and vitamin E (Tan, Huang, & Qu, 1998; Zhao, Alexeev, Chang, Greenburg, & Bojanowski, 2005). Traditionally, *L. barbarum* is described as having the properties of nourishing the blood, enriching the yin, tonifying the kidney and liver, and moistening the lungs. It is applied in the treatment of such conditions as consumptive disease accompanied by thirst (includes early-onset diabetes and tuberculosis), dizziness, blurred vision, diminished visual acuity, and chronic cough (Yu et al., 2005).

Polysaccharides, extracted from traditional Chinese herb *L. barbarum*, can fortify the immune system and has been found to be a powerful secretagogue (a substance that stimulates the secretion of rejuvenative human growth hormone by the pituitary gland) (Luo et al., 2006; Zhang et al., 2005). Studies have shown that the *L. barbarum* polysaccharide, the effective compound of *L. barbarum* fruit, can efficiently reduce senescing and improve blood function in middle-aged and elderly people, and in trial experiments it showed a marked inhibitory activity on tumor cells (Cao et al., 1994; Qian, Cheung, & Richardson, 1989; Xu, Xu, & An, 2000). At the present time, it is being prescribed as antitumor, anticancer, antioxidant, hypoglycemic, immunological activities, anti-senescence drug and as a means to improve the immunological competence of the human body (Shi, Jia, & Dong, 1997; Song & Yang, 1997; Xu et al., 2000). Both in vitro and in vivo studies, the results showed that LBP was an effective anticancer compound. In vivo, LBP could dose-dependently decrease tumor weight and increase the amount of splenocytes, proliferation of activated T cells, NK activity and TNF-α levels in S180-bearing mice (Liu, Zhang, & Qian, 1996). In vitro, 20–1000 mg/L LBP could inhibit the growth of human leukemia HL-60 cells in dose-dependent manner and decrease the membrane fluidity (Gan, Zhang, Yang, & Xu, 2004).

However, efficient production of *L. barbarum* polysaccharides has been a bottleneck in all their promising applications. The objective of this present study was to optimize the process for production of *L. barbarum* Polysaccharides, using response surface methodology (RSM), employing a three-level, four-variable Box–Behnken design.

* Corresponding author. Tel/fax: +86 898 66895004.
E-mail address: hn.yingh@yahoo.com.cn (G. Yin)

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doi:10.1016/j.carbpol.2008.04.025

2. Materials and methods

2.1. Materials

Fruits of *L. barbarum*, family *solanacae*, originated from china were purchased from local herb market (HaiKou, China).

All other chemicals and solvents used were of analytical grade.

2.2. Methods

2.2.1. Preparation of polysaccharides

Polysaccharides from *L. barbarum* were prepared by the method of Luo, Cai, Yan, Sun, and Corke (2004). The dried fruit samples (10 g) were ground to fine powder and immersed into a given volume of hot water and the extraction process was performed with different temperature and time. The extract was left to cool at room temperature, filtered, and then freeze-dried to obtain crude polysaccharides. The dried crude polysaccharides were refluxed three times to remove lipids with 150 ml of chloroform:methanol solvent (2:1) (v/v). After filtering the residues were air-dried. The result product was extracted several times in a given volume of hot water and then filtered. The combined filtrate was precipitated using 150 ml of 95% ethanol, 100% ethanol and acetone, respectively. After filtering and centrifuging, the precipitate was collected and freeze-dried under vacuum using a Micromodulyo freeze-drier (Thermo Savant, USA), giving desired polysaccharides.

The content of the polysaccharides was measured by phenolsulfuric method (Masuko et al., 2005).

2.2.2. Experimental design

Box–Behnken statistical screening design was used to statistically optimize the formulation parameters and evaluate main effects, interaction effects and quadratic effects of the formulation ingredients on the yield of *L. barbarum* polysaccharides. After determining the significant factors, the optimum operation conditions are attained by using more complex experimental designs such as Doehlert matrix (DM), central composite designs (CCD) and three-level designs such as the Box–Behnken design (BBD) (Chopra et al., 2007; Hou & Chen, 2008; Cai, Gu, & Tang, 2008). A 4-factor, three-level design used is suitable for exploring quadratic response surfaces and constructing second-order polynomial models with SAS (Version 8.0, SAS Institute, Cary, NC, USA). The Box–Behnken design was specifically selected since it requires fewer runs than a CCD in cases of three or four variables. This cubic design is characterized by set of points lying at the midpoint of each edge of a multidimensional cube and center point replicates ($n = 3$) whereas the 'missing corners' help the experimenter to avoid the combined factor extremes. This property prevents a potential loss of data in those cases (Box & Behnken, 1960). For statistical calculations, the relation between the coded values and actual values are described as the following equation:

$$X_i = (A_i - A_0)/\Delta A, \tag{1}$$

where X_i is a coded value of the variable; A_i the actual value of variable; A_0 the actual value of the A_i at the center point; and ΔA the step change of variable.

A design matrix comprising of 27 experimental runs was constructed. The non-linear computer-generated quadratic model is given as

$$Y = \beta_0 + \sum_{i=0}^{4} \beta_i X_i + \sum_{j=0}^{4} \beta_{ii} X_i^2 + \sum_{i=0}^{4} \sum_{j=0}^{4} \beta_{ij} X_i X_j, \tag{2}$$

where Y is the measured response associated with each factor level combination; β_0 is an intercept; β_i is regression coefficients computed from the observed experimental values of Y; and X_i is the coded levels of independent variables. The terms X_iX_j and X_i^2 represent the interaction and quadratic terms, respectively. The dependent and independent variables selected are shown in Table 1 along with their low, medium, and high levels, which were selected based on the results from preliminary experimentation. The concentration range of ratio of water to raw material (X_1), extraction temperature (X_2), extraction time (X_3), and number of extraction (X_4) used to prepare the 27 formulations and the respective observed responses are given in Table 2.

2.3. Analysis of monosaccharide composition of L. barbarum polysaccharides

Purified polysaccharide sample was hydrolyzed in 2 M HCl for 2.5 h at 105 °C in a sealed glass tube. The residual acid was removed under vacuum, followed by co-distillation with water. Then the hydrolyzates were converted to acetylated aldononitrile derivatives according to conventional protocols and analyzed by the TRACE GC 2000 gas chromatograph (Finnigan, Italy) using an OV-1701 capillary column (30 m × 0.32 mm ID). As references, the following neutral sugars were converted to their acetylated derivatives and analyzed: rhamnose, arabinose, xylose, mannose, glucose, and galactose (Shi, Sheng, Yang, & Hu, 2007).

Table 1
Variables and experimental design levels for response surface

Independent variables	Coded symbols	Levels		
		−1	0	1
Ratio of water to raw material	X_1	27	30	33
Extraction temperature	X_2	90	95	100
Extraction time	X_3	4.5	5	5.5
Number of extraction	X_4	4	5	6

Table 2
The Box–Behnken experimental design with four independent variables

No.	X_1 (ratio of water to raw material)	X_2 (extraction temperature, °C)	X_3 (extraction time, h)	X_4 (number of extraction)	Extraction yield (%)
1	−1 (27)	−1 (90)	0 (5)	0 (5)	12.5
2	−1 (27)	1 (100)	0 (5)	0 (5)	20.3
3	1 (33)	−1 (90)	0 (5)	0 (5)	13.8
4	1 (33)	1 (100)	0 (5)	0 (5)	21.5
5	0 (30)	0 (95)	−1 (4.5)	−1 (4)	14.4
6	0 (30)	0 (95)	−1 (4.5)	1 (6)	17.2
7	0 (30)	0 (95)	1 (5.5)	−1 (4)	18.4
8	0 (30)	0 (95)	1 (5.5)	1 (6)	19.8
9	−1 (27)	0 (95)	0 (5)	−1 (4)	15.8
10	−1 (27)	0 (95)	0 (5)	1 (6)	17.9
11	1 (33)	0 (95)	0 (5)	−1 (4)	16.5
12	1 (33)	0 (95)	0 (5)	1 (6)	19.8
13	0 (30)	−1 (90)	−1 (4.5)	0 (5)	10.2
14	0 (30)	−1 (90)	1 (5.5)	0 (5)	15.5
15	0 (30)	1 (100)	−1 (4.5)	0 (5)	20.2
16	0 (30)	1 (100)	1 (5.5)	0 (5)	22.3
17	−1 (27)	0 (95)	−1 (4.5)	0 (5)	15.9
18	−1 (27)	0 (95)	1 (5.5)	0 (5)	19.3
19	1 (33)	0 (95)	−1 (4.5)	0 (5)	18.1
20	1 (33)	0 (95)	1 (5.5)	0 (5)	19.8
21	0 (30)	−1 (90)	0 (5)	−1 (4)	12.8
22	0 (30)	−1 (90)	0 (5)	1 (6)	14.1
23	0 (30)	1 (100)	0 (5)	−1 (4)	19.9
24	0 (30)	1 (100)	0 (5)	1 (6)	20.6
25	0 (30)	0 (95)	0 (5)	0 (5)	19.4
26	0 (30)	0 (95)	0 (5)	0 (5)	19.4
27	0 (30)	0 (95)	0 (5)	0 (5)	19.4

3. Results and discussion

3.1. Effect of extraction time on yield of polysaccharides

Extraction time is an important parameter of the *L. barbarum* polysaccharides extraction (Hou & Chen, 2008). Extraction time is not constant during the extraction stages. Here, extraction time was, respectively, set at 3, 3.5, 4, 4.5, 5, 5.5, and 6 h to examine the influence of extraction time on the yield of the polysaccharides extraction when other reaction conditions were as follows: extraction temperature 95 °C, ratio of water to raw material 33, and number of extraction 6. As shown in Fig. 1a, the yield of the polysaccharides increased with the increase of extraction time. The polysaccharides achieved a maximum percentage of 20.7% when the extraction time was 5.5 h. After this point, the yield of the polysaccharides started to maintain a dynamic equilibrium with increasing the extraction time. Therefore, a 5.5-h extraction time was adopted in the work.

3.2. Effect of extraction temperature on yield of polysaccharides

According to the researches of Oosterveld, Beldman, Schols, and Voragen (1996) and Zykwinska, Rondeau-Mouro, Garnier, Thibault, and Ralet (2006), increasing of extraction yield is realized by increasing constant temperature bath time period. Fig. 1b shows this effect of extraction temperature on yield of polysaccharides. In this research, extraction temperature as an important extraction parameter was set at 70, 75, 80, 85, 90, 95, and 100 °C to investigate the influence of extraction temperature on the yield of the polysaccharides when other extraction conditions were similar to those described in Section 3.1. According to Fig. 1b, a similar trend in results can be observed at the given temperature conditions. The extraction yield increases up to its maximum amount at 95 °C. The yield of polysaccharides no longer increased when extraction temperature continued to rise.

3.3. Effect of ratio of water to raw material on yield of polysaccharides

Different ratio of water to raw material will significantly affect extract yield (Govender et al., 2005). If ratio of water to raw material is too small, polysaccharides in raw material cannot be completely extracted up. If ratio of water to raw material is too big, this will cause high process cost. Therefore, suitable ratio of water to raw material should be selected for extraction of targeted polysaccharides. In the present studies, ratio of water to raw material as an important extraction parameter was set at 18, 21, 24, 27, 30, 33, and 36 to investigate the influence of different ratio of water to raw material on the yield of the polysaccharides when other extraction conditions were similar to those described in Section 3.1. As be shown in Fig. 1c, the polysaccharides yield increase with increasing ratio of water to raw material, and reaches highest value when the ratio is 33. A possible explanation is that increase in ratio of water to raw material may increase diffusivity of the solvent into cells and enhance desorption of the polysaccharides from the cells (Ray, 2004; Volpi, 2005). However, the results from the present experiments indicated that when the ratio is higher than 33, the polysaccharides yield was decreased. This might be due to the reason that polysaccharides could be excessively dissolved in lower concentration solvent, making big loss during production collection. Therefore, ratio of water to raw material 33 was adopted in the work.

3.4. Effect of number of extraction on yield of polysaccharides

The number of extraction is also an important factor which influences the yield of polysaccharides during heat water extraction (Ferreira, Mafra, Rosário Soares, Evtuguin, & Coimbra, 2006). The samples were extracted with water for different number of extraction (2, 3, 4, 5, and 6) (Fig. 1d) when other extraction conditions were similar to those described in Section 3.1. The results showed that the yield of polysaccharides has obvious increase with

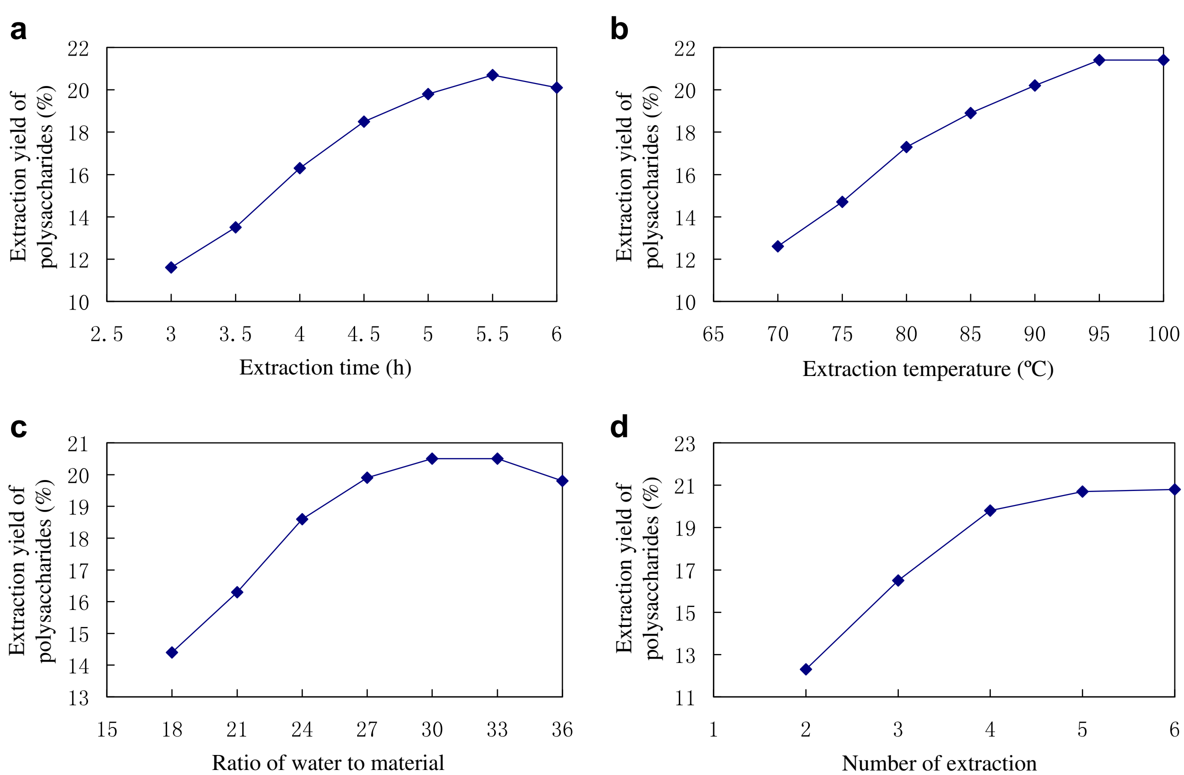

Fig. 1. Effect of different extraction parameters (extraction time, h; extraction temperature, °C; ratio of water to raw material; and number of extraction) on yield of polysaccharides.

Table 3
Fit statistics for Y

	Master model	Predictive model
Mean	17.58519	17.58519
R^2	98.39%	98.39%
Adj. R^2	96.51%	96.51%
RMSE	0.575483	0.575483
CV	3.272544	3.272544

the number of extraction (2–6). The yield of polysaccharides was the biggest when number of extraction was 6. This result indicates that a number of extraction of 6 is enough to the polysaccharides in the present work.

3.5. Optimization of the yield of the polysaccharides extract

Response surface optimization is more advantageous than the traditional single parameter optimization in that it saves time,

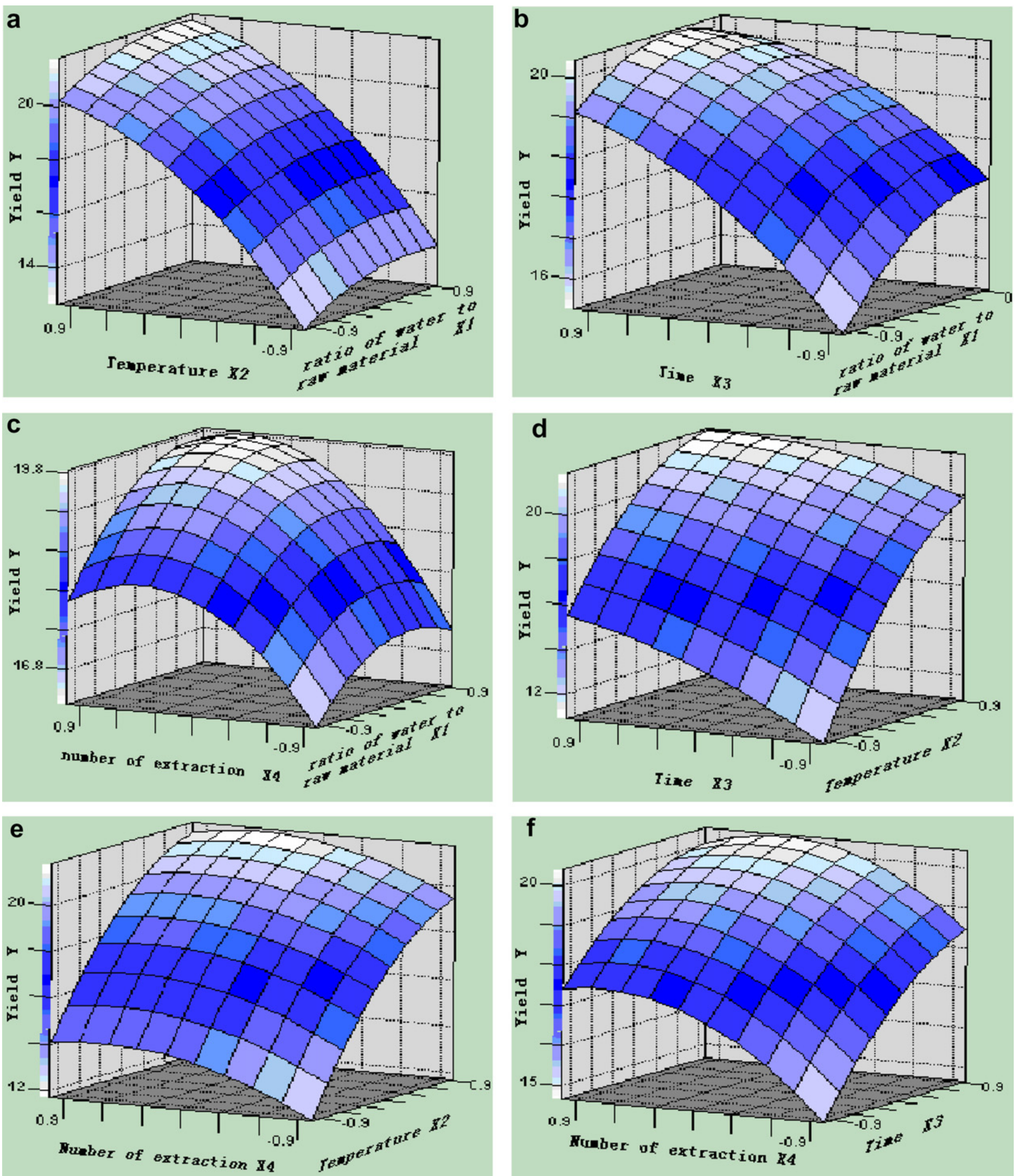

Fig. 2. Response surface (3D) showing the effect of different extraction parameters (X_1: ratio of water to raw material; X_2: extraction temperature, °C; X_3: extraction time, h; and X_4: number of extraction) added on the response Y.

space and raw material. There were a total of 27 runs for optimizing the four individual parameters in the current Box–Behnken design. The current design was applied to the production of polysaccharides from *L. barbarum* by heat water extraction. The data were analyzed by multiple regression analysis using the SAS version 8.0 and the following polynomial equation was derived to represent polysaccharides yield as a function of the independent variables tested. Where Y is the predicted polysaccharides acid yield and X_1, X_2, X_3, and X_4 are the coded values for extraction time, extraction temperature, ratio of water to raw material and number of extraction, respectively. Table 2 shows the experimental conditions and the results of polysaccharides production according to the factorial design. Maximum yield of polysaccharides (22.3%) was recorded under the experimental conditions of ratio of water to raw material 30, extraction temperature 100 °C, extraction time 5.5 h and number of extraction 5.

The experimental data were statistically analyzed using the SAS package for analysis of variance (ANOVA) and the results are shown in Table 3. The ANOVA of the quadratic regression model showed that the value of the determination coefficient (R^2 = .9839), indicating that only 1.1% of the total variations were not explained by the model. The value of the adjusted determination coefficient (Adj R^2 = .9651), which also confirmed that the model was highly significant. At the same time, a relatively lower value of the coefficient of variation (CV = 3.27%) indicated a better precision and reliability of the experiments carried out.

$$Y = 19.4 + 0.65 * X_1 + 3.825 * X_2 + 1.591667 * X_3 + 0.966667 * X_4 - 0.658333 * X_1 * X_1 - 0.025 * X_1 * X_2 - 0.425 * X_1 * X_3 + 0.3 * X_1 * X_4 - 1.595833 * X_2 * X_2 - 0.8 * X_2 * X_3 - 0.15 * X_2 * X_4 - 0.670833 * X_3 * X_3 - 0.35 * X_3 * X_4 - 1.158333 * X_4 * X_4. \quad (3)$$

The coefficient estimate for the parameter optimization suggested that all the independent variables studied (X_1, X_2, X_3, X_4) and four quadratic terms (X_1^2, X_2^2, X_3^2, X_4^2) significantly affected the polysaccharides yield. The analysis also showed that there were significant interactions between extraction temperature and ratio of water to raw material. The results of the study showed that the extraction temperature was the most significant single parameter which influenced polysaccharides yield followed by ratio of water to raw material and number of extraction (Table 2; Eq. (4)). Neglecting the insignificant terms, the final predictive equation obtained is as given below:

$$Y = 19.4 + 0.65 * X_1 + 3.825 * X_2 + 1.591667 * X_3 + 0.966667 * X_4 - 0.658333 * X_1 * X_1 - 1.595833 * X_2 * X_2 - 0.8 * X_2 * X_3 - 0.670833 * X_3 * X_3 - 1.158333 * X_4 * X_4. \quad (4)$$

Response surfaces were plotted using SAS version 8.0 software to study the effects of parameters and their interactions on polysaccharides yield. Three-dimensional response surface plots and two-dimensional contour plots, as presented in Figs. 2a–f and 3a–f, are very useful to see interaction effects of the factors on the responses. These types of plots show effects of two factors on the response at a time. In all the presented figures, the other two factors were kept at level zero.

As expected, a greater increase in polysaccharides yield resulted when the extraction temperature (X_2) was increased in the range from 90 to 100 °C. The temperature curve did not level off at 100 °C, which may indicate that a slightly higher temperature is required to achieve maximum increase (Figs. 2a and 3a). Likewise, a great increase in polysaccharides yield resulted when the ratio of water to raw material (X_1) was increased in the range from 27 to 33. The ratio curve started to level off at 33, which may indicate that a ratio of water to raw material of 33 (X_1) is required to achieve maximum increase (Figs. 2a and 3a).

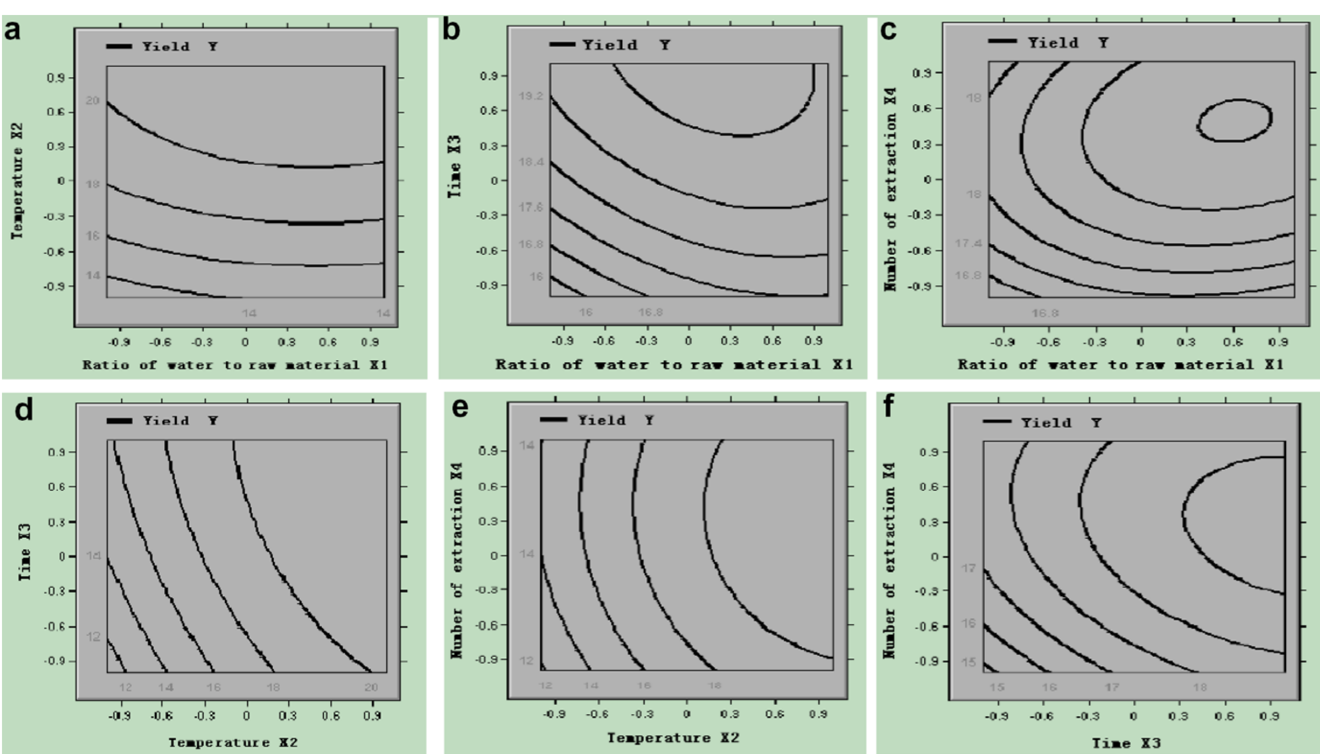

Fig. 3. Contour plots (2D) showing the effect of different extraction parameters (X_1: ratio of water to raw material; X_2: extraction temperature, °C; X_3: extraction time, h; and X_4: number of extraction) added on the response Y.

Table 4
Optimum conditions, predicted and experimental value of response at that condition

Optimum condition				Yield of polysaccharides (%)	
Extraction time (h)	Extraction temperature (°C)	Ratio of water to raw material	Number of extraction	Experimental	Predicted
2.8	50	40	3	22.56 ± 1.67	23.13

Figs. 2b and 3b show that extraction time (X_3) exhibited a significant effect whereas ratio of water to raw material (X_1) showed a weaker effect on the polysaccharides yield. The yield of polysaccharides extracted increased with extraction time (X_3) and ratio of water to raw material (X_1) (Figs. 2b and 3b). Increased ratio of water to raw material (X_1) up to a threshold level of 31.2 led to increased polysaccharides yield. Beyond this level, polysaccharides yield slightly decreased (Figs. 2b and 3b).

As ratio of water to raw material (X_1) was increased in the range from 27 to 31.2, polysaccharides yield increased. At low ratio of water to raw material (X_1) levels, the curve did not level off, indicating that 31.2 is well below optimum for polysaccharides yield. Yield of polysaccharides have a ratio of water to raw material (X_1) optimum of 31.2. A low level of number of extraction (X_4) had a stimulatory effect on polysaccharides yield. When number of extraction (X_4) is 5, maximum yield of polysaccharides was achieved after which yield of polysaccharides no longer markedly changed with increasing number of extraction (Figs. 2c and 3c).

As in the case of polysaccharides extract, extraction temperature (X_2) and extraction time (X_3) used both had a positive impact on the polysaccharides production. There was a linear increase in the yield of polysaccharides with increase in the extraction temperature (X_2) and extraction time (X_3) (Figs. 2d and 3d). The contours were slightly inclined to the horizontal showing that there was a significant interaction between the two parameters. Thus, it may be said that a higher level of extraction temperature (X_2) and extraction time (X_3) is required to achieve maximum increase of polysaccharides yield (Figs. 2d and 3d).

The 3D response surface based on independent variables extraction temperature (X_2) and number of extraction (X_4) was shown in Fig. 2e, while the other two independent variable, ratio of water to raw material (X_1) and extraction time (X_3) was kept at a zero level. The interaction relationship between the two independent variables extraction temperature (X_2) and number of extraction (X_4) can be easily understood by examining the contour plots generated by keeping the other two independent variable, ratio of water to raw material (X_1) and extraction time (X_3) as constant (Fig. 3e).

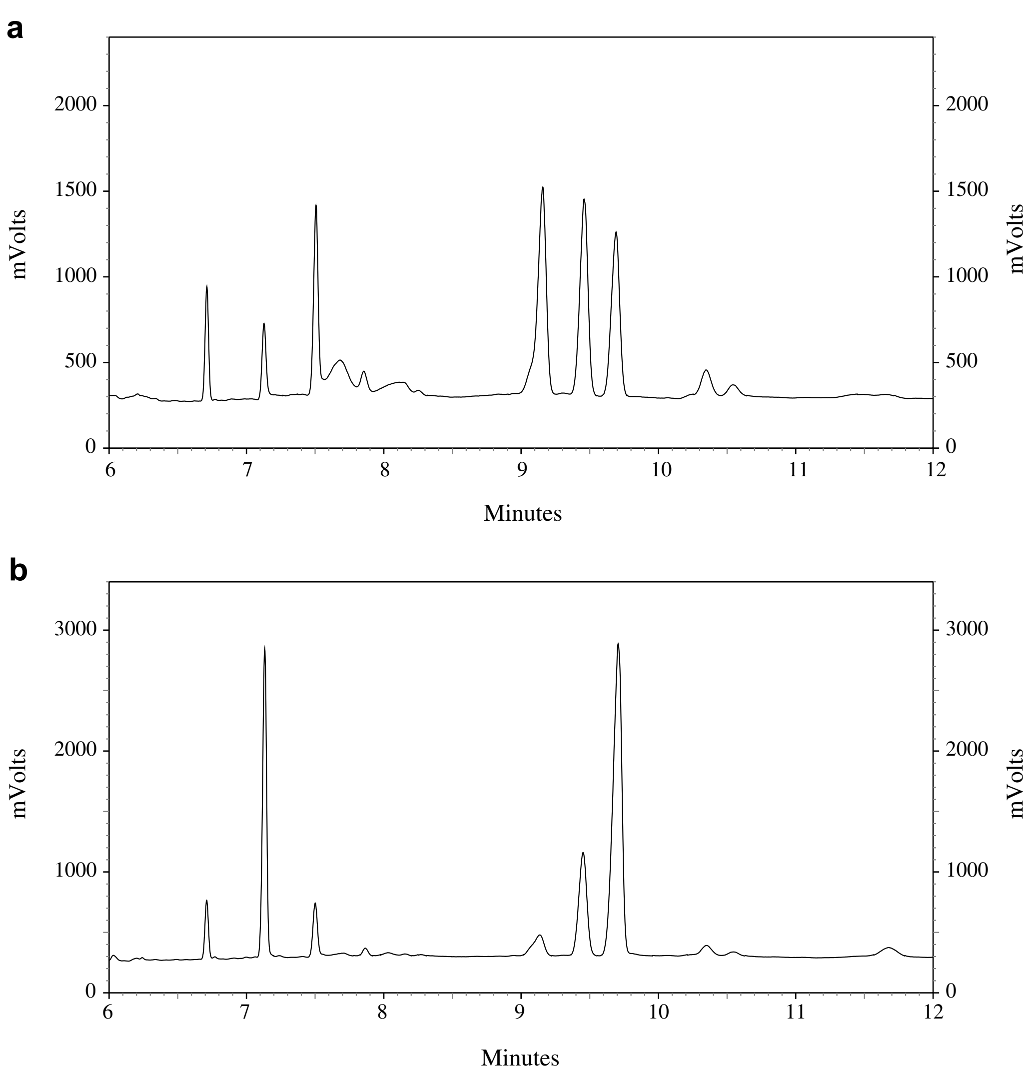

Fig. 4. (a) GC analysis of standard samples. (b) GC analysis of polysaccharides.

An increase in the yield of polysaccharides could be significantly achieved with the increases of extraction temperature (X_2). It was obvious that the yield of polysaccharides was increase with the increase in number of extraction (X_4) from 4 to 6, meaning that further increases of number of extraction (X_4) would not increase the yield of polysaccharides any longer (Figs. 2e and 3e).

Figs. 2f and 3f show the effect of interaction of extraction time (X_3) and number of extraction (X_4) on the yield of polysaccharides. A increase in yield was observed with increase in number of extraction (X_4) from 4 to 6, which was in good agreement to the above mention single-factor test (Fig. 1d). A single parameter study would overlook this entity. An interaction of extraction time (X_3) and number of extraction (X_4) was no obvious as temperature was a factor that influenced the yield of polysaccharides. It was obvious that the yield of polysaccharides was increase with the increase in extraction time (X_3) from 4.5 to 5.5, meaning that a longer extraction time (X_3) is required to achieve maximum increase of the yield of polysaccharides (Fig. 2f and Fig. 3f).

3.6. Validation of the models

In order to validate the adequacy of the model equations (Eqs. (3) and (4)), a verification experiment was carried out under the optimal conditions (within the experimental range): extraction time 5.5, extraction temperature 100 °C, ratio of water to raw material 31.2, number of extraction 5. Under the optimal conditions, the model predicted a maximum response of 23.13 (%). To ensure the predicted result was not biased toward the practical value, experimental rechecking was performed using this deduced optimal condition. A mean value of 22.56 ± 1.67 (%) ($N = 4$), obtained from real experiments, demonstrated the validation of the RSM model. The good correlation between these results confirmed that the response model was adequate for reflecting the expected optimization (Table 4). The results of analysis indicated that the two groups of experimental values were in good agreement with the predicted ones, and also suggested that the models of Eqs. (3) and (4) are satisfactory and accurate.

3.7. Characterization of polysaccharides

The total sugar content of the crude polysaccharide was determined to be 95.1% with a purity >98.3%. It had no absorption at 280 or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid.

GC traces of the polysaccharide hydrolyzates (Fig. 4b), compared with standard saccharides (rhamnose, arabinose, xylose, mannose, glucose, and galactose) (Fig. 4a) showed that the monosaccharide components of the samples were rhamnose, arabinose, xylose, mannose, galactose, and glucose with a mole ratio of 0.31:2.05:0.26:0.41:1:3.14 (Fig. 4b). This was in basic agreement with result of Li et al.'s work (2006).

4. Conclusion

The response surface method proved to be useful for optimization of technology of polysaccharides extraction. Statistical analysis proved to be a useful and powerful tool in developing optimum extraction conditions. The statistical analysis based on a Box–Behnken design showed that an extraction time of 5.5 h, an extraction temperature of 100 °C, an ratio of water to raw material of 31.2 and an number of extraction of 5 were the best conditions to produce *L. barbarum* polysaccharides. Under the most suitable conditions, maximum yield of polysaccharides 23.13% can be achieved. Analysis of the polysaccharides showed that it was a homogeneous polysaccharide, with a weight-average molecular weight of 156,000 Da. GC analysis indicated that the polysaccharides consisted of xylose, mannose, arabinose, rhamnose, glucose, galactose with a mole ratio of 0.31:2.05:0.26:0.41:1:3.14.

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