African Journal of Biotechnology Vol. 10(75), pp. 17241-17250, 28 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2270
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Optimization of ultrasonic extraction of mycelial polysaccharides from *Paecilomyces hepiali* using response surface methodology and its antioxidant activity

Shi-jun Yu[1]*, Ying Zhang[1], Chun-ru Li[1], Qian Zhang[1], Zhong-you Ma[1,2] and Mei-zhen Fan[1]*

[1]Anhui Provincial Key Laboratory of Microbial Control, Anhui Agricultural University, Hefei, Anhui, 230036, China.
[2]Anhui University of Science and Technology, Fengyang, Anhui, 233100, China.

Accepted 17 October, 2011

Ultrasonic technology was applied to extract mycelial polysaccharides from *Paecilomyces hepiali* and the process was optimized by response surface methodology. Antioxidant activity of polysaccharides was also investigated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (%DPPHsc). Three independent variables were ratio of water to raw material (x_1), ultrasonic power (x_2) and extraction time (x_3). Statistical analysis indicated that x_1, x_2, x_1^2, x_3^2, x_1x_2, x_1x_3 and x_2x_3 had significant effect on the yields. Besides, x_2, x_1^2 and x_2^2 shaped the %DPPHsc of polysaccharides significantly. Three dimensional surface plots and contour plots were drawn from the mathematical model. The optimal conditions for yield and %DPPHsc were as follows: condition (1) x_1 = 125 mL/g, x_2 = 300 W, x_3 = 12 min, and condition (2) x_1 = 125 mL/g and x_2 = 500 W, x_3 = 11 min, respectively. Under these conditions, the yield was 9.37%, and %DPPHsc was 45.34%. There existed good agreement between experimental and predicted values.

Key words: Ultrasonic extraction, polysaccharides, *Paecilomyces hepiali*, response surface methodology, antioxidant activity.

INTRODUCTION

Cordyceps spp. is a class of rare and exotic medicinal fungi, and it has been used as traditional medicine and health food in China and other Asia countries for thousands of years (Holliday et al., 2005). Many Cordyceps have been studied and reported with the effect of anti-oxidant, anti-aging, immune modulating, anti-cancer and anti-inflammation (Li and Tsim, 2004; Li et al., 2003; Paterson, 2008). *Paecilomyces hepiali* was firstly isolated from fresh fruiting body of *Cordyceps sinensis* (synonym of *Ophiocordyceps sinensis*), of which a close relationship with *C. sinensis* was reported (Dai et al., 1989). The mycelial powder of *P. hepiali* has been intensively studied and developed into functional food in China for many years. Polysaccharides, adenosine and cordycepin in the mycelial powder of *P. hepiali* are considered as the major functional compositions for the health effects (Holliday and Cleaver, 2008; Miyazaki et al., 1977; Wasser, 2002).

As a major class of biomolecules, polysaccharides are the most complex and least appreciated for their bioactivities for a long time (Berg et al., 2002). Over recent years, the bioactive polysaccharides from fungi, lichens, higher plants and animal resources have gained great attention, especially in food and drug industries throughout the world (Chang, 2002; Forabosco et al., 2006). They play a vital role in the growth and development of all living organisms, and have been extensively studied due to their unique biological, chemical and physical properties (Schepetkin and Quinn, 2006).

*Corresponding author. E-mail: shijun.yu@hotmail.com, mzfan.44@163.com

Abbreviations: RSM, Response surface methodology; **RCEF,** Research Center for Entomogenous Fungi; **BBD,** Box-Behnken design**; DPPH,** 2,2-diphenyl-1-picrylhydrazyl.

However, up till now, there are no detail investigations and literatures available on systematical study of the extraction of polysaccharides from the mycelia powder of *P. hepiali*. As it is known, hot-water extraction is a conventional extraction technology of polysaccharides but it usually requires high temperature and consumes long time; and the extraction efficiency is low (Li et al., 2007). Therefore, it is necessary to establish a high efficient and economical extraction technology of polysaccharides from the mycelia powder *of P. hepiali.*

In recent years, ultrasonic extraction has attracted numerous attention and been employed widely in the extraction of valuable compounds from biomass since it has many advantages such as working at room temperature, lower cost and higher efficiency than the conventional extraction methods (Hromádková and Ebringerová, 2003; Hromádková et al., 2002; Mason et al., 1996; Vilkhu et al., 2008; Wang et al., 2009). The great extraction efficiency of ultrasonic treatment is mainly attributed to its mechanical effects, which facilitates mass transfer between immiscible phases through a super agitation (Vinatoru et al., 1997), especially, microjetting and microstreaming (Tsochatzidis et al., 2001; Velickovic et al., 2006; Zhong and Wang, 2010; Zou et al., 2011).

Response surface methodology (RSM) is an effective mathematics and statistics technique to optimize and analyze complex process (Montgomery, 2000). Box-Behnken design, one of RSM, has been widely used by researchers and only has three levels with fewer experimental trials. Thus, it is easier to arrange and interpret experiments (Box and Behnken, 1960; Claver et al., 2010; Ferreira et al., 2007). In addition, it is less laborious and time-consuming than other methods to optimize a process. It has been widely used in food engineering, pharmaceuticals, bioprocessing agrochemicals and other industries to extract biological materials, such as polysaccharides, phenolic compounds and protein from various sources (Cai et al., 2008; Hou and Chen, 2008; Qiao et al., 2009; Wang et al., 2005; Xiao et al., 2004; Zhao et al., 2009; Zhu et al., 2010).

In the current study, the ultrasonic extraction condition of polysaccharides from *P. hepiali* mycelia was firstly investigated and optimized by RSM. The antioxidant activity of the polysaccharides was also evaluated *in vitro* by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity.

MATERIALS AND METHODS

Microorganism

A strain of *P. hepiali* RCEF0936 was isolated from fresh fruiting bodies of *C. sinensis* and provided by Research Center for Entomogenous Fungi (RCEF) of Anhui Agricultural University. The strain, identified by Prof. Z.Z. Li (Anhui Agricultural University) and Dr. Y.L. Guo (Institute of Microbiology, Chinese Academy of Sciences), is deposited at RCEF of Anhui Agricultural University.

Culture of mycelial powder

The mycelial powder was cultured by liquid submerged fermentation in Anhui Provincial Key Laboratory of Microbial Control of Anhui Agricultural University. The fermented mycelia were harvested from the broth by centrifugation at 16000 rpm for 30 min. The gained mycelia were dried at 45°C to a constant weight by hot air dryer and ground into powder by a muller (A11 basic, ZKA-WERKE, Germany) and stored at 4°C refrigerators.

Extraction of polysaccharides

According to previous experimental experience, the ultrasonic extraction process was performed at different ratios of water to raw material (x_1: 100-160 mL/g), ultrasonic power (x_2: 300-450 W), and extraction time (x_3: 8-14 min). The suspension was then centrifuged (5000 rpm, 10 min) and supernatant was precipitated by the addition of ethanol to a final concentration of 75% (v/v). Precipitates were collected by centrifugation (5000 rpm, 10 min), washed successively with ethanol, ethylether and acetone and then dissolved in deionized water. The content of polysaccharides were determined by phenol-sulfuric acid colorimetric method (Dubois et al., 1956). And the yield of polysaccharides was calculated by the equation following:

$$\text{Yield of polysaccharides}\,(\%) = \frac{\text{weight of polysaccharides}\,(\text{g})}{\text{weight of raw material}\,(\text{g})} \times 100\% \quad (1)$$

Design of experiments

Based on our previous single-factor experiments for the extraction of polysaccharides, the Box-Behnken design (BBD) was employed to determine the best combination of the extraction variables for the production of polysaccharides. The level and code of variables, which were considered in this study are shown in Table 1. The average of triplicates values of each run was taken as dependent variables or response. The variables were coded according to the equation as following,

$$x_i = (x_i - x_0)/\,_{\Delta}x \quad (2)$$

Where, x_i is the (dimensionless) coded value of the variable x_i; x_0 is the value of x_i at the center point and $_{\Delta}x$ is the step change. Based on the experimental data, regression analysis was performed and fitted into an empirical quadratic polynomial model:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} x_i x_j \quad (3)$$

Where, Y is the response variable; β_0, β_i, β_{ii}, β_{ij} are the regression coefficients of variables for intercept, linear, quadratic and interaction terms, respectively and x_i and x_j are the independent variables ($i \neq j$). The model evaluated the effect of each independent variable to a response. Design expert (Version 8.0.4.1 trial, State-Ease inc., Minneapolis, USA) was employed for the experimental design, data analysis, quadratic model building and graph (three dimensional response surface and contour) plotting.

Assay of (1,1-diphenyl-2-picryl-hydrazyl) DPPH radical scavenging activity

The antioxidant activity of the polysaccharides was determined by

Table 1. Independent variables and their levels in the response surface design.

Variables	x_j	factor levels		
		-1	0	+1
Ratio of water to raw material (ml/g)	x_1	100	130	160
Ultrasonic power (W)	x_2	300	450	600
Extraction time (Min)	x_3	8	11	14

Table 2. BBD matrix and the responses of the dependent variables (actual and predicted).

Run	Coded variable levels			Yield (%)		%DPPHsc (%)	
	x_1	x_2	x_3	Actual	Predicted	Actual	Predicted
1	0	0	0	8.99	8.57	37.09	42.99
2	-1	-1	0	7.02	6.97	21.16	23.61
3	0	0	0	8.66	8.57	45.27	42.99
4	0	0	0	8.47	8.57	46.02	42.99
5	0	0	0	8.27	8.57	47.61	42.99
6	-1	0	-1	5.04	5.34	29.84	30.67
7	0	+1	+1	4.36	4.62	34.17	37.45
8	0	0	0	8.45	8.57	38.98	42.99
9	+1	-1	0	9.48	9.69	21.26	23.84
10	+1	+1	0	6.36	6.41	31.64	29.19
11	+1	0	+1	7.42	7.12	27.36	26.53
12	0	+1	-1	6.54	6.45	41.29	41.05
13	-1	+1	0	6.38	6.17	36.98	36.40
14	0	+1	0	6.63	6.37	30.31	27.03
15	-1	0	+1	4.35	4.31	35.27	32.57
16	+1	0	-1	5.45	5.50	25.04	27.74
17	0	-1	+1	8.70	8.79	31.08	31.32

DPPH radical scavenging test according to the method of Hu et al. (2004) with some modification. Samples were diluted to the concentration of 50 µg/mL prior to analysis. One hundred microlitre of each sample was thoroughly mixed with 100 µL of 0.4 mg/ml freshly prepared DPPH. The absorbance was measured at 517 nm for 20 min at 25 ºC in the dark. Distilled water was used as the control. The scavenging activity of DPPH radicals by the sample was calculated according to the following equation:

$$\text{Scavenging percentage activity } (\%) = (1 - \frac{A_1 - A_2}{A_0}) \times 100\% \quad (4)$$

Where, A_0 is the absorbance of the control (water instead of polysaccharides solution); A_1 is the absorbance of the sample and A_2 is the absorbance of the sample under identical conditions as A_1 with water instead of DPPH solution.

RESULTS AND DISCUSSION

The effect of three process variables such as ratio of raw material, ultrasonic power and extraction time were studied during the extraction of polysaccharides from the mycelia of *P. hepiali*. Two responses of interest were extraction yield and DPPH radical scavenging activity. The results of 17 runs using BBD are presented in Table 2 that includes the design, experimental responses and predicted values. The results demonstrate that there was a good agreement between the experimental and predicted values. And the yields ranged from 4.35 to 9.48%. The maximum yield (9.48%) was found under the extraction conditions of x_1 = 160 mL/g, x_2 = 300.00 W, x_3 = 11 min. On the other hand, the antioxidant property (%DPPHsc) ranged from 21.16 to 47.61%. The highest %DPPHsc (47.61%) value was in the condition of x_1 = 130 mL/g, x_2 = 450 W, x_3 = 11 min. It seemed that these conditions varied depending on the response required. Therefore, the optimal ultrasonic extraction condition should be investigated in order to achieve high extraction yield and antioxidant activity.

Model fitting

The significance of each coefficient was checked using F-test and p-value as shown in Table 3 and the p-value also

Table 3. Analysis of variance for response surface quadratic model of the yield of polysaccharides.

Source	Sum of squares	df	Mean square	F-value	p-value
Yield (%)[c]					
Model	42.32	9	4.70	44.75	<0.0001 [b]
Quadratic	21.57	3	7.19	68.42	<0.0001 [b]
x_1	4.39	1	4.39	41.75	0.0003 [b]
x_2	8.38	1	8.38	79.79	<0.0001 [b]
x_3	0.17	1	0.17	1.61	0.2446
x_1x_2	1.54	1	1.54	14.63	0.0065 [b]
x_1x_3	1.76	1	1.76	16.79	0.0046 [b]
x_2x_3	4.51	1	4.51	42.96	0.0003 [b]
x_1x_1	5.33	1	5.33	50.69	0.0002 [b]
x_2x_2	0.076	1	0.076	0.72	0.4231
x_3x_3	14.86	1	14.86	141.43	<0.0001 [b]
Residual	0.74	7	0.11		
Lack of fit	0.44	3	0.15	1.97	0.2605
Total	43.06	16			
%DPPHsc (%)[d]					
Model	914.10	9	101.57	5.20	0.0205 [a]
Quadratic	645.35	3	215.12	11.01	0.0048 [b]
x_1	40.29	1	40.29	2.06	0.1942
x_2	202.80	1	202.80	10.38	0.0146 [a]
x_3	0.24	1	0.24	0.012	0.9148
x_1x_2	7.41	1	7.41	0.38	0.5575
x_1x_3	2.41	1	2.41	0.12	0.7357
x_2x_3	15.59	1	15.59	0.80	0.4014
x_1x_1	423.84	1	423.84	21.69	0.0023 [b]
x_2x_2	113.81	1	113.81	5.82	0.0466 [a]
x_3x_3	54.01	1	54.01	276	0.1404
Residual	136.81	7	19.54		
Lack of fit	50.19	3	16.73	0.77	0.5668
Total	1050.92	16			

[a]significant at 0.05 level, [b]significant at 0.01 level, [c]The coefficient of the determination (r^2) of the model was 0.9829, [d]The coefficient of the determination (r^2) of the model was 0.8698.

indicated the interaction strength of each parameter. The smaller the p-value, the more significant the corresponding coefficient is (Murthy et al., 2000). The results of analysis of variance (ANOVA) indicated that the contribution of quadratic model was significant for responses of extraction yield and antioxidant activity. The fitted quadratic models for extraction yield and %DPPHsc in codes variables are given in Equation 5 and 6, respectively.

$$\text{Extraction yield (\%)} = 8.57+0.74x_1-1.02x_2+0.15x_3-0.62x_1x_2+0.66x_1x_3-1.06x_2x_3-1.12x_1^2-0.13x_2^2-1.88x_3^2 \quad (5)$$

$$\text{DPPHsc (\%)} = 42.99-2.24x_1+5.03x_2+0.17x_3-1.36x_1x_2-0.78x_1x_3-1.97x_2x_3-10.03x_1^2-5.20x_2^2-3.58x_3^2 \quad (6)$$

Extraction yield

It can be observed that the variable with the largest effect on the extraction yield was linear term of ultrasonic power (x_2) and quadratic term of extraction time (x_3^2) and followed by quadratic term of ratio of water to raw material (x_1^2), the interaction of ultrasonic power and extraction time (x_2x_3), the linear term of ratio of water to raw material (x_1), the interaction terms x_1x_3 and x_1x_2. The results shown in Table 3 suggested that the change of ratio of water to raw material and ultrasonic power had significant effect ($p < 0.01$) on the yield. The coefficient of determination (R^2) was 0.9829, implying that the sample variation of 98.29% was attributed to the variables and less than 2% of the total variance could not be explained

by the model. Therefore, the present R^2 value reflected an excellent fitness between the experimental and the predicted response, and indicated that the model is reliable for extraction of polysaccharides in the present study. These values would give a relative good fit to the mathematic model in Equation 5.

Antioxidant activity (%DPPHsc)

In the light of antioxidant activity, it can be found that the quadratic term of ratio of water to raw material (x_1^2) gave the greatest contribution and followed by linear term of ultrasonic power (x_2) and quadratic term of ultrasonic power (x_2^2). The coefficient of determination (R^2) of the predicted models in this response was 0.8698 and p-value for Lack of fit was 0.5668, suggesting a good fitness to the mathematical model (Equation 6). The predicted models can represent the experimental values.

Interpretation of response surface model and contour plots

The response surface and contour plots are the graphical representations of regression equation. They provide visual interpretation of 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, indicated whether the reciprocal interactions between the variables are significant or not. The circular contour plot indicates that the interactions between corresponding variables are negligible, while elliptical contour plot indicated that the interactions between corresponding variables are significant (Muralidhar et al., 2001).

Extraction yield

In the current study, three independent response surface plots and their respective contour plots were generated by design expert as shown in Figure 1. It can be seen that the extraction yield increased considerably with the increase of ratio of water to raw material from 100 to 126.7 mL/g and then decreased slightly as shown in Figure 1a and b. However, the effect of ultrasonic power displayed a potential decrease at the experimental range. With regard to the effect of extraction time, similar change pattern with ratio of water to raw material is shown in Figure 1c and d. Maximum extraction yield was obtained at middle level of ratio of water to raw material and extraction time when ultrasonic power at a constant level at 300 W. Figure 1e and f show the interaction of ultrasonic power and extraction time on extraction yield. We can see that ultrasonic power almost had no impact on extraction yield from 300 to 600 W and the extraction yield increased with the increase of extraction time from 8 to 12.1 min and decreased from 12.1 to 14 min.

Antioxidant activity (%DPPHsc)

Figure 2 shows the response surface plots and contour plots for the present study and depicted the pair-wise interaction of the three variables on scavenging activity of DPPH free radical while maintaining the other variables at their zero levels. It was evident that the extracted polysaccharides consisted of different antioxidant property by DPPH assay. Figure 2a and b show the response surface plot and contour plot at varying ratio of water to raw material and ultrasonic power at fixed extraction time (0 level). From these two figures, we can conclude that the %DPPHsc increased with the increase with ratio of water to raw material from 100 to 125.80 mL/g and then decreased from 125.80 to 160 mL/g. And the %DPPHsc was found to increase rapidly with the increase of ultrasonic power from 300 to 517.18 W, then decreased slightly from 517.18 to 600 W. The %DPPHsc affected by different ratio of water to raw material and extraction time are shown in Figure 2c and d when ultrasonic power was fixed at 0 level. It can be seen that the %DPPHsc reached the maximum value when ratio of water to raw material and extraction time at the threshold levels of 125.80 mL/g and 10.7 min, respectively. In Figure 2e and f, the response surface plot and the contour plot were developed for the %DPPHsc of polysaccharides with varying ultrasonic power and extraction time at fixed ratio of water to raw material (0 level). It indicated that the maximum %DPPHsc value can be achieved when ultrasonic power and extraction time were 517.18 W and 10.7 min, respectively.

From Figure 2, it can be concluded that the optimal extraction condition of polysaccharides from *P. hepialid* for high %DPPHsc is the ratio of water to raw material of 125.80 mL/g, ultrasonic power of 528 W and extraction time of 10.7 min.

Validation of predicted model

To further validate optimal values, first partial derivatives of regression equation were taken and made to be zero. Calculating the Equation 4 gave the optimal conditions for the extraction of polysaccharides as follows: x_1 = 125 mL/g, x_2 = 300 W, and x_3 = 12 min. The theoretical yield of polysaccharides under the above conditions was 9.46%. To test validity of the response surface analysis method, polysaccharides was extracted under the optimal conditions and the yield of polysaccharides was 9.37% (n = 3), which was in accordance with the predicted values significantly ($p > 0.05$). The results indicate that the experimental design model was adequate for the extraction of water-soluble polysaccharides from the mycelial

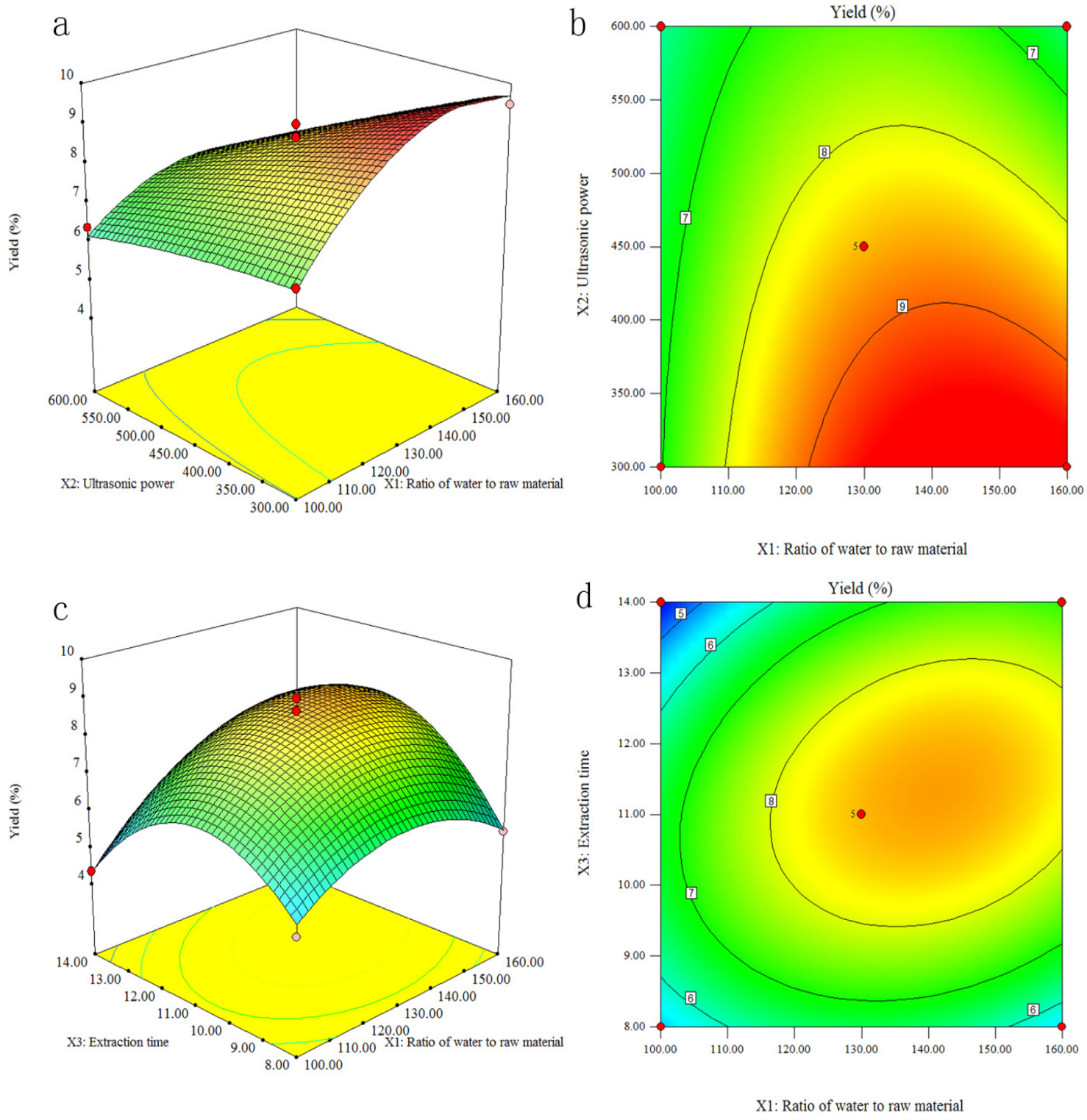
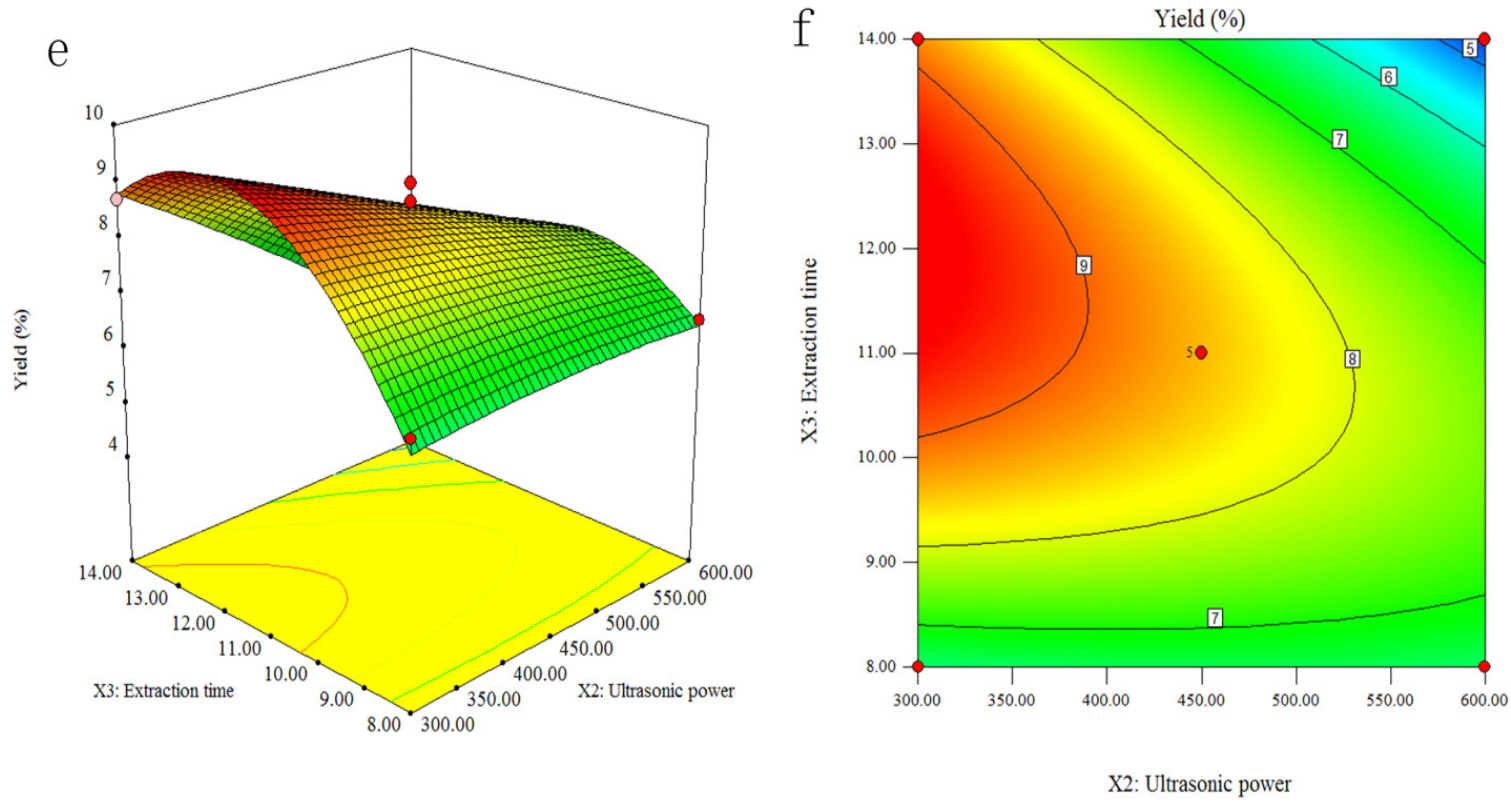


Figure 1. Response surface plots (a, c, and e) and contour plots (b, d and f) showing the effect of ratio of water to raw material (x_1), ultrasonic power (x_2) and extraction time (x_3) on the yield of polysaccharides.

powder of *P. hepiali.*

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of natural compounds (Chen et al., 2008). According to the regression analysis of Equation 6, the optimal conditions for the extraction of poly-



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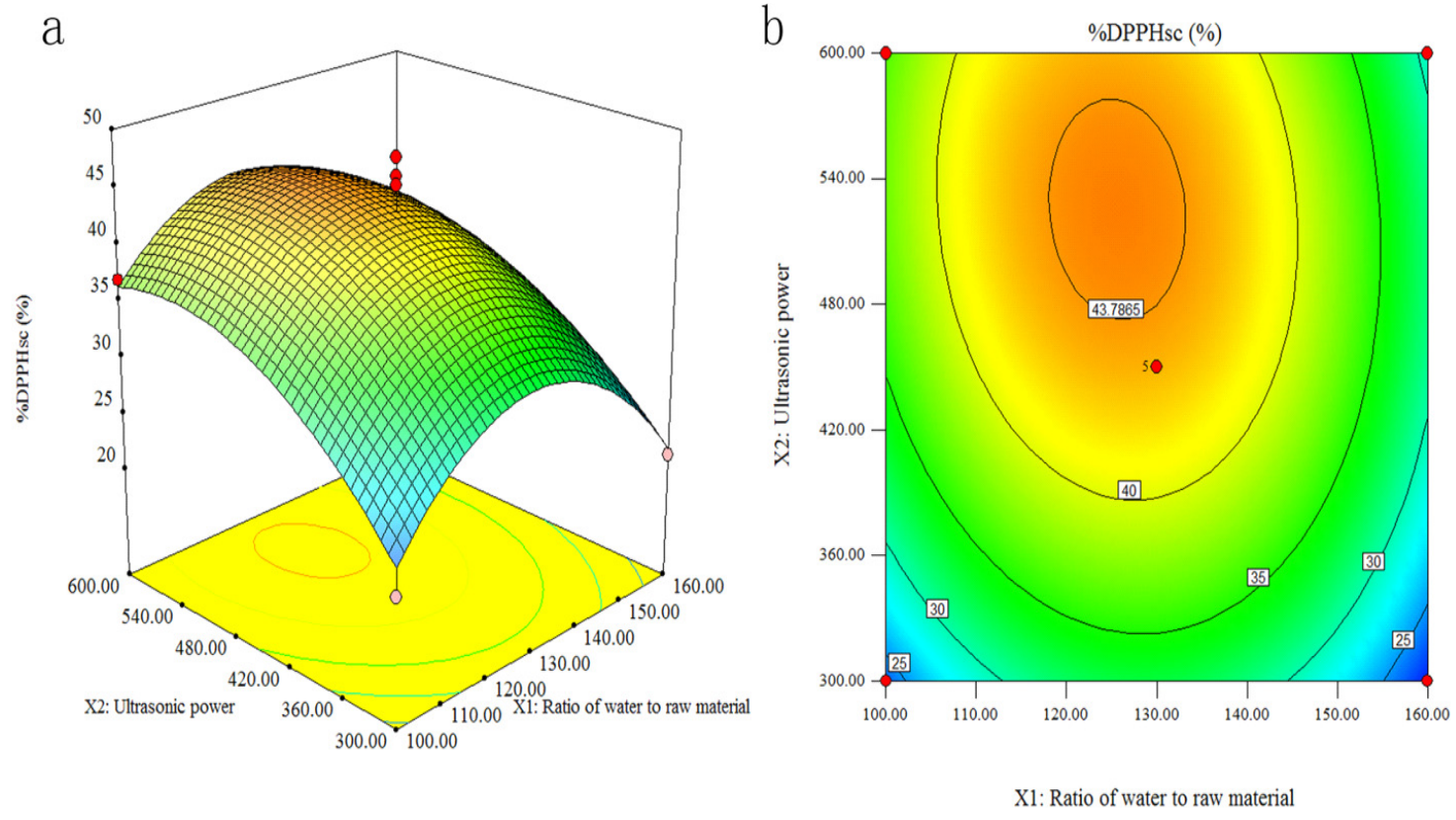

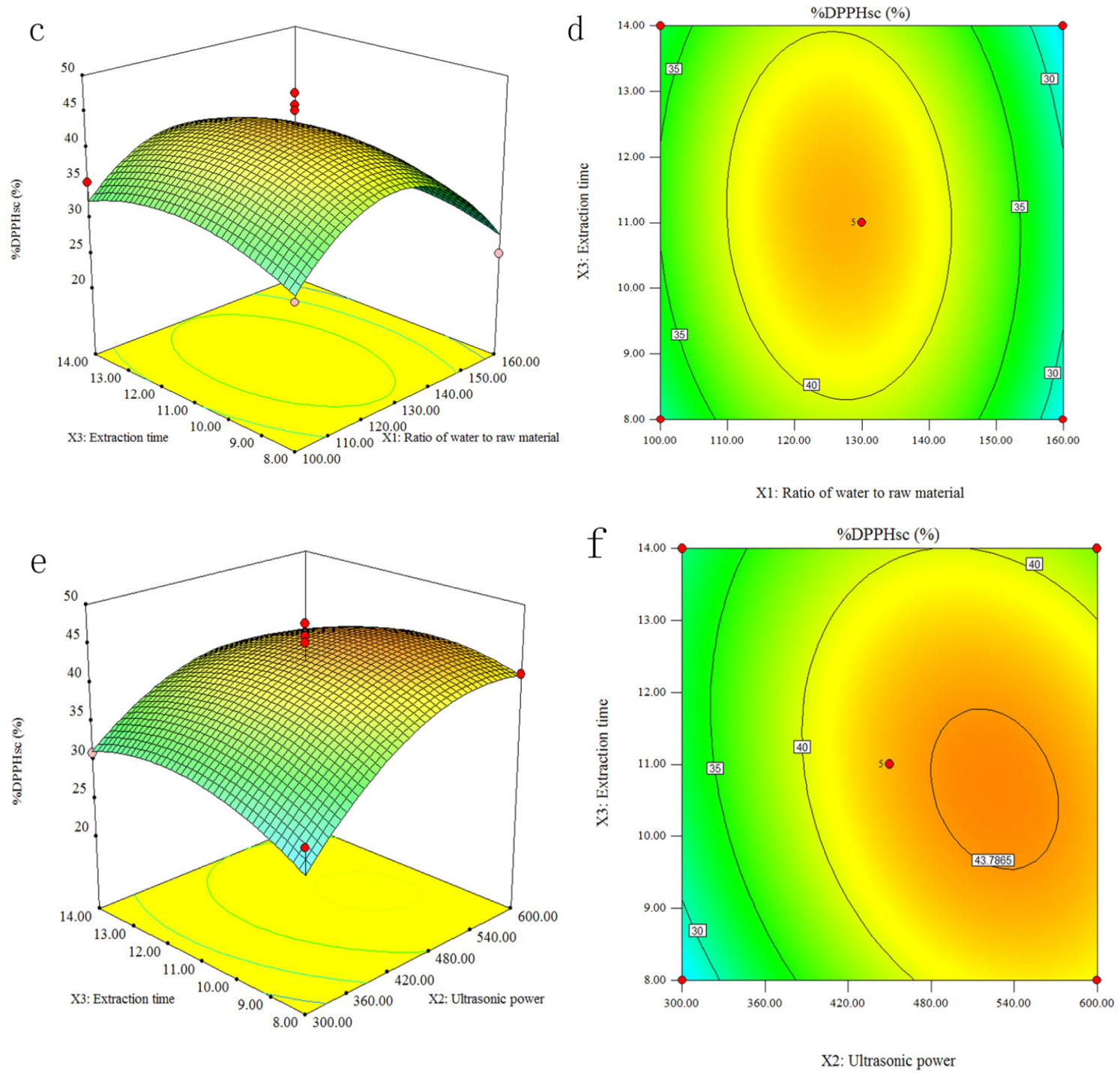

Figure 2. Response surface plots (a, c, and e) and contour plots (b, d and f) showing the effect of ratio of water to raw material (x_1), ultrasonic power (x_2) and extraction time (x_3) on %DPPHsc.

saccharides as follows: x_1 = 125 mL/g, x_2 = 500 W, and x_3 = 11 min. The best predicted DPPH radical scavenging activity of polysaccharides was 45.34%. Some researchers studied the polysaccharides from the rind from *Punica granatum*, *Turbinaria ornate, Bryopsis plumose, Ganoderma lucidum*, respectively and found that the studied various polysaccharides had the activities of scavenging DPPH radicals (Ananthi et al., 2010; Chen et al., 2009; Rout and Banerjee, 2007; Song et al., 2010). The results show that there was a difference between the optimized conditions of yield and DPPH scavenging activity. This may attribute to the difference of poly-

saccharides fractions with different DPPH scavenging capacity at different extraction conditions.

Conclusions

Ultrasonic technology was performed for the polysaccharides extraction from mycelial powder of *P. hepiali*. RSM was used to estimate and optimize the experimental variables of ratio of water to raw material (ml/g), ultrasonic power (W) and extraction time (min). The optimal extraction conditions for the yield of polysaccharides were determined as following: ratio of water to raw material 125 ml/g, ultrasonic power 300 W and extraction time 12 min. Under these conditions, the experimental yield of polysaccharides was 9.37 ± 0.29%, which was agreed closely with the predicted value. And the optimal conditions for %DPPHsc were ratio of water to raw material of 125 ml/g, ultrasonic power of 500 W and extraction time of 11 min. The %DPPHsc was 45.34 ± 2.49% under these conditions, which was in good agreement with the predicted values 44.46%.

ACKNOWLEDGEMENT

This work was supported by the National High Technology Research and Development Program of China (863 Program) (No. 2007AA021506).

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