Optimization of Extraction Process, Analysis of Polysaccharides from *Morchella septimelata*

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Abstract *Morchella septimelata* polysaccharide (MSP) possesses multiple biological activities. In this study, double enzyme method was used to extract polysaccharides from *Morchella septimelata*. The basis of single factor experiment and response surface design was used to optimize the extraction process of polysaccharides. The optimal extraction conditions of polysaccharide were as follows: extraction time 2 h, extraction temperature 40.5 ℃, solid-liquid ratio 1 : 15 (*w/v*), and the amount of double enzyme (cellulase: β-glucanase, mass ratio 1 : 2) 2.0 %. Under these conditions, the extraction yield of polysaccharide reached (14.86 ± 0.12)%.

Key words *Morchella septimelata*; Polysaccharides extraction; Response surface design
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Morchella septimelata is a filamentous fungus, belonging to Ascomycota family, and is one of the four most famous edible fungi in the world, which is very popular with consumers because of its delicious taste and rich nutrition[1-2]. Modern scientific research has found that *M. septimelata* polysaccharide (MSP) has anti-tumor[3], anti-fatigue[4], antioxidation[5], and antibacterial activity[6] among others. Hence, it has become more and more attractive in the field of polysaccharide research.

At present, the main extraction methods of MSP include hot water extraction, ultrasonic-assisted extraction, enzymatic extraction, and extraction methods under osmotic pressure and high-voltage pulse electric field. The method of hot water extraction is easy to operate and requires relatively simple equipment[7], but it has the disadvantage of low extraction yield. The method of ultrasonic assisted extraction[8] has the advantages of short time, high yield and saving energy, but the power of ultrasonic wave needs to be well controlled and not too high, otherwise it could readily destroy the structures of glycoproteins, resulting in an unpredictable loss of biological activity or even detrimental effects due to the change of primary and secondary structure or even composition or configuaration. Osmotic pressure method[9] has the advantage of low cost, high yield and easier separation of the obtained product for further processing. The method with high voltage pulse electric field[10] has no heat generation and does not destroy the structural characteristics of polysaccharides, but its extraction process is complex and requires large-scale instruments. Compared with other methods, enzymatic extraction[11], which utilizes enzymes to disrupt cellular structure and allow the efficient dissolution of intracellular polysaccharides, has the characteristics of good specificity, high yield and no damage to polysaccharide structure. Wu *et al.*[12] used β-glucanase from *Aspergillus niger* to extract poria polysaccharide, and under optimal conditions, the yield of polysaccharide could reach 12.8%. Due to the limited efficiency and yield of single enzyme extraction method, coupled enzyme methods have been developed in recent years, with higher extraction efficiency than single-step enzyme method. Xu *et al.*[13] tested the extraction effect of cellulase, hemicellulase and β-glucanase and concluded that the extraction rate of poria polysaccharide was as high as 16.13% under the optimal conditions. However, the use of cellulase and β-glucanase to extract MSP and optimize the extraction process are still relatively scarce.

Improving the yield of MSP has great prospects in the field of functional foods and medicinal purpose. In order to improve the extraction efficiency of *M. septimelata*, this study used response surface methodology (RSM) to optimize the extraction process of *M. septimelata*. The best extraction conditions of MSP were studied using four single factors (extraction time, temperature, ratio of solid material to liquid, enzyme dosage) and RSM. The work establishes a foundation for optimizing the extraction conditions of MSP and offers a reference and guidance for the development and application of its bioactive properties.

Materials and Methods

Reagents Materials

M. septimelata fruiting body was purchased from Hubei Jinding Ecological Agriculture Co, Ltd. (Macheng, Hubei province,

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Ruiling LYU (1982 -), P. R. China, devoted to research about development of rare edible and medicinal fungal resources.
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China). Concentrated sulfuric acid, aqueous hydrogen peroxide (30%), trichloroacetic acid, glucose, ferrous sulfate, ferric chloride, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), and salicylic acid were purchased from Sinopharm Chemical Reagent Co, Ltd. (Shanghai, China). Vitamin C standard (purity 99%) was purchased from Shanghai Jinsui Bio-Technology Co., Ltd. (Shanghai, China). Cellulase (20 U/mg) and β-glucanase (100 U/mg) were purchased from Zhejiang Yinuo Biotechnology Co., Ltd. (Lanxi, Zhejiang province, China).

Preparation of glucose reference solution

Glucose (0.1 g) was added to 100 ml of distilled water to obtain a stock solution (1.0 mg/ml). Control solution of glucose (0.1 mg/ml) was prepared by 10-fold dilution of the above stock solution[14].

Determination of the polysaccharide content by phenol-sulfuric acid method

Phenol-sulfuric acid method[15-17] was used to determine the content of polysaccharides. First, 1 ml of each 8 different glucose concentrations was prepared by mixing 0, 100, 200, 300, 400, 500, 600 and 700 μl of 0.1 mg/ml glucose control solution with distillated water, respectively. Then, 5% of phenol solution (1 ml) and concentrated sulfuric acid (5 ml) was added to each of the above 1 ml of 8 glucose solutions, then heated in a 30 ℃ water bath for 30 min after gently mixing for 10 min. Using distilled water as blank control, the absorbance of each resulted solution was measured at 490 nm. The glucose concentration (mg/ml) was used as the abscess coordinate, and the absorbance value was used as the coordinate to draw a standard curve. Curvilinear regression equation was obtained as $Y = 0.0097X - 0.0586$ with $R^2 = 0.995$. The content of polysaccharide was calculated based on the standard curve after the absorbance measurement of an extracted sample of crude polysaccharide extracts. The extraction rate of polysaccharide was calculated according to the following formula.

$$\text{Extraction Rate} = (C \times V_0 \times N)/(V_1 \times M) \quad (1)$$

In the above formula, C is the concentration of polysaccharide after dilution (mg/ml); V_0 is the liquid volume of polysaccharide extraction (ml); N is the dilution (ml); V_1 is the volume of sample tested (ml); M is the mass of dried polysaccharide (mg).

Single factor experiment

Different extraction time was first evaluated at 30, 60, 90, 120, 150 and 180 min, respectively, under the condition of other parameters unchanged. Same analog, extraction temperatures (25, 30, 35, 40, 45, and 50 ℃), solid-liquid ratio (1 : 5, 1 : 10, 1 : 15, 1 : 20, 1 : 25, and 1 : 30) and enzyme dosage (0.5, 1, 2, 3, 4 and 5 mg) were evaluated independently while keeping other conditions constant.

Response surface optimization experiment

The response surface method was used in the optimization of extraction efficiency of polysaccharides. According to the results of single-factor experiment, four factors including extraction time (X_1), temperature (X_2), ratio of solid to liquid (X_3) and enzyme dosage (X_4) were selected as independent variables, and the extraction yield (Y) was the response value of polysaccharides. RSM was used to design an experiment with four factors at three levels. The experimental factors and levels are shown in Table 1.

Table 1 Factors and levels of Box-Behnken design

Independent Variables	Levels		
	-1	0	1
Extraction time (h) (X_1)	1.5	2	2.5
Extraction temperature (℃) (X_2)	35	40	45
Solid-liquid ratio (g : ml) (X_3)	1 : 10	1 : 15	1 : 20
Enzyme dose (X_4)	1	2	3

Data analysis

All data were denoted as mean standard deviation (*SD*) of three replicates. One-way ANOVA analysis and Duncan's multiple range test ($P < 0.05$) were performed using SPSS 22.0. The graphs were plotted using Origin 8.0 software. Microsoft Excel 2016 was used to process data and mapping.

Results and Discussion

Effects of four single factors on extraction yield of polysaccharides

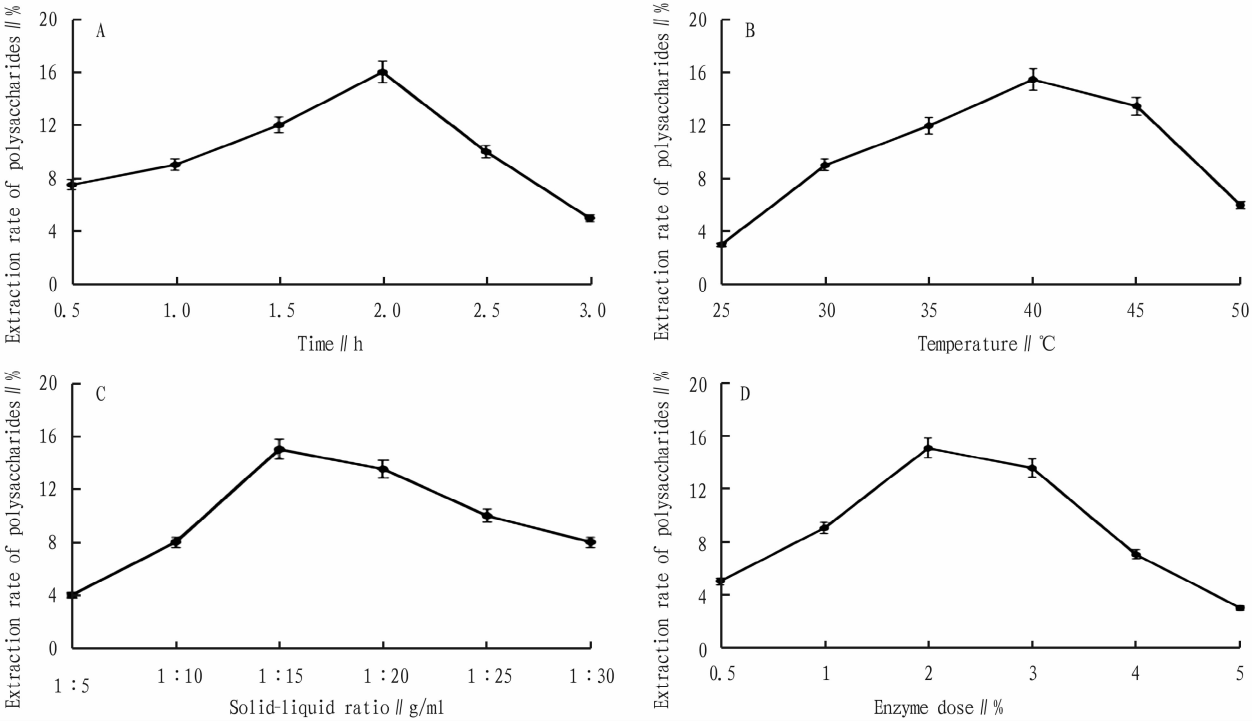
Extraction time Extraction time is a crucial factor in the extraction process. Short time often results in low extraction yield, but prolonged time more often also gives low yield, and high energy cost and low production efficiency. Optimization of extraction time is of great importance. In the extraction of polysaccharide from *M. esculenta* as shown in Fig. 1A, with the increase of extraction time from 0.5 - 2 h, the yield of polysaccharide also increased. When the extraction time was 2 h, the extraction rate of MSP reached 16%, but it decreased to 10 % when the extraction time was more than 2 h. Therefore, 2 h was selected as the optimized extraction time of polysaccharide.

Extraction temperature Temperature variation has direct impact on the yield of extraction of bioactives. As shown in Fig. 1B, with the increase of extraction temperature, the yield of MSP also increased. When the extraction temperature reached 40 ℃, the maximum extraction yield was 15.5%, then decreased to 13.5% when the temperature reached 45 ℃. Therefore, 40 ℃ was the optimized extraction temperature of MSP.

Solid-liquid ratio The ratio of solid to extraction solvent is also an important factor. As shown in Fig. 1C, with the increase of solid-liquid ratio, the yield of polysaccharide also increased. When the solid-liquid ratio was 1 : 15, the polysaccharide extraction rate reached maximum at 15%. When the solid-liquid ratio continued to increase beyond 1 : 15 while other factors were kept constant, the extraction yield of polysaccharide decreased. Considering that too much material to liquid ratio will cause solvent waste andit is not conducive to post-treatment, the solid-liquid ratio of 1 : 15 was considered to be more suitable for this experiment.

Enzyme dosage In a certain range, the yield of polysaccharide increased with the increase of enzyme dose. As shown in Fig. 1D, when the enzyme dose reached 2%, the polysaccharide extraction rate was 15%, but when the enzyme dose was higher than 2%, the polysaccharide extraction rate decreased. Therefore, the enzyme dose of 2% was the optimized enzyme dose of MSP.

According to the above results, extraction time 1.5, 2.0, and 2.5 h, extraction temperature 35, 40, and 45 ℃, solid-liquid ratio 1 : 10, 1 : 15 and 1 : 20, and enzyme dose 1%, 2% and 3% were chosen for RSM experiments.



(A) Extraction time; (B) Extraction temperature; (C) Solid-liquid ratio; (D) Enzyme dose.

Fig. 1 Effects various factors on extraction rate of polysaccharides from *M. esculenta*

Statistical analysis and model fitting

On the basis of the above single factor test, a Box-Behnken design (BBD) was used to investigate the effects of extraction time, extraction temperature, solid-liquid ratio, and enzyme dose on the extraction yield of polysaccharide. The experimental conditions of 29 runs and the extraction yields of polysaccharide are shown in attachment Table 1. The yield of polysaccharide ranged from 8.07% to 16.08%. The quadratic multinomial regression equation was obtained as follows:

$$Y = 15.16 + 0.46X_1 - 0.74X_2 - 0.19X_3 + 1.53X_4 + 1.3X_1X_2 + 0.32X_1X_3 - 1.33X_1X_4 + 0.88X_2X_3 - 0.56X_2X_4 - 0.24X_3X_4 - 1.40X_1^2 - 2.55X_2^2 - 3.15X_3^2 - 2.04X_4^2$$

Where Y is the yield of polysaccharide (%), X_1 is extraction time (h), X_2 is extraction temperature (℃), X_3 is solid-liquid ratio (w/v), and X_4 is enzyme dose (%).

The results of polysaccharide extraction yield were analyzed by multiple regression analysis. As shown in Table 2, the F value of the model was 12.40 and $P < 0.01$, indicating that the model was significant[18]. The determination coefficient (R^2) and the adjusted determination coefficient (R^2_{adj}) were 0.925 4 and 0.850 8, respectively, implying that the model had a good fitting degree with the actual yield of polysaccharide extraction and could be used to predict the best conditions for the extraction of polysaccharide. At the same time, the linear term (X_1, X_2) and interaction term (X_1X_2, X_1X_4) was significant ($P < 0.05$), and the linear term (X_4) and quadratic term (X_1^2, X_2^2, X_3^2, X_4^2) was extremely significant ($P < 0.01$). Other terms were considered non-significant ($P > 0.05$).

We used Design expert 8.0.6 software to draw three-dimensional (3D) response surface to study the effects of parameters and their interaction on the yield of polysaccharide. According to the regression equation, the response surface and contour plots of the yield of polysaccharide with the change of each factor are drawn in Fig. 2. The results of 3D response surface analysis showed that the maximum extraction yield of polysaccharide could be found in the range of this experiment. The enzyme dose had the greatest impact on the extraction yield of polysaccharide, followed by extraction temperature, extraction time, and solid-liquid ratio. The interactions between enzymolysis temperature and time (Fig. 2A), solid-liquid ratio and enzymolysis time (Fig. 2B), enzyme dose and enzymolysis time (Fig. 2C), solid-liquid ratio and enzymolysis temperature (Fig. 2D), enzyme dose and enzymolysis temperature (Fig. 2E), enzyme dose and solid-liquid ratio (Fig. 2F) on the yield of polysaccharide could be easily obtained. It showed that these factors affected each other, which were consistent with the results of ANOVA.

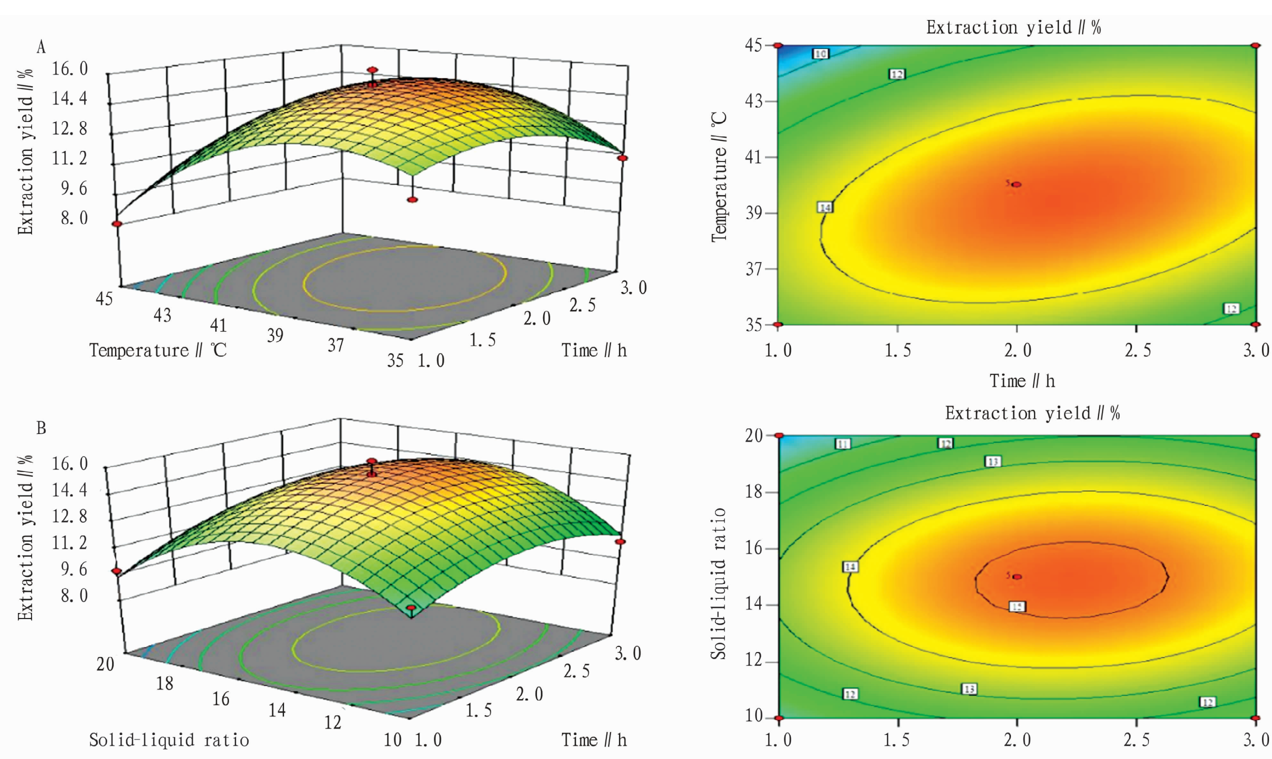
Table 2 Box-Behnken variance analysis

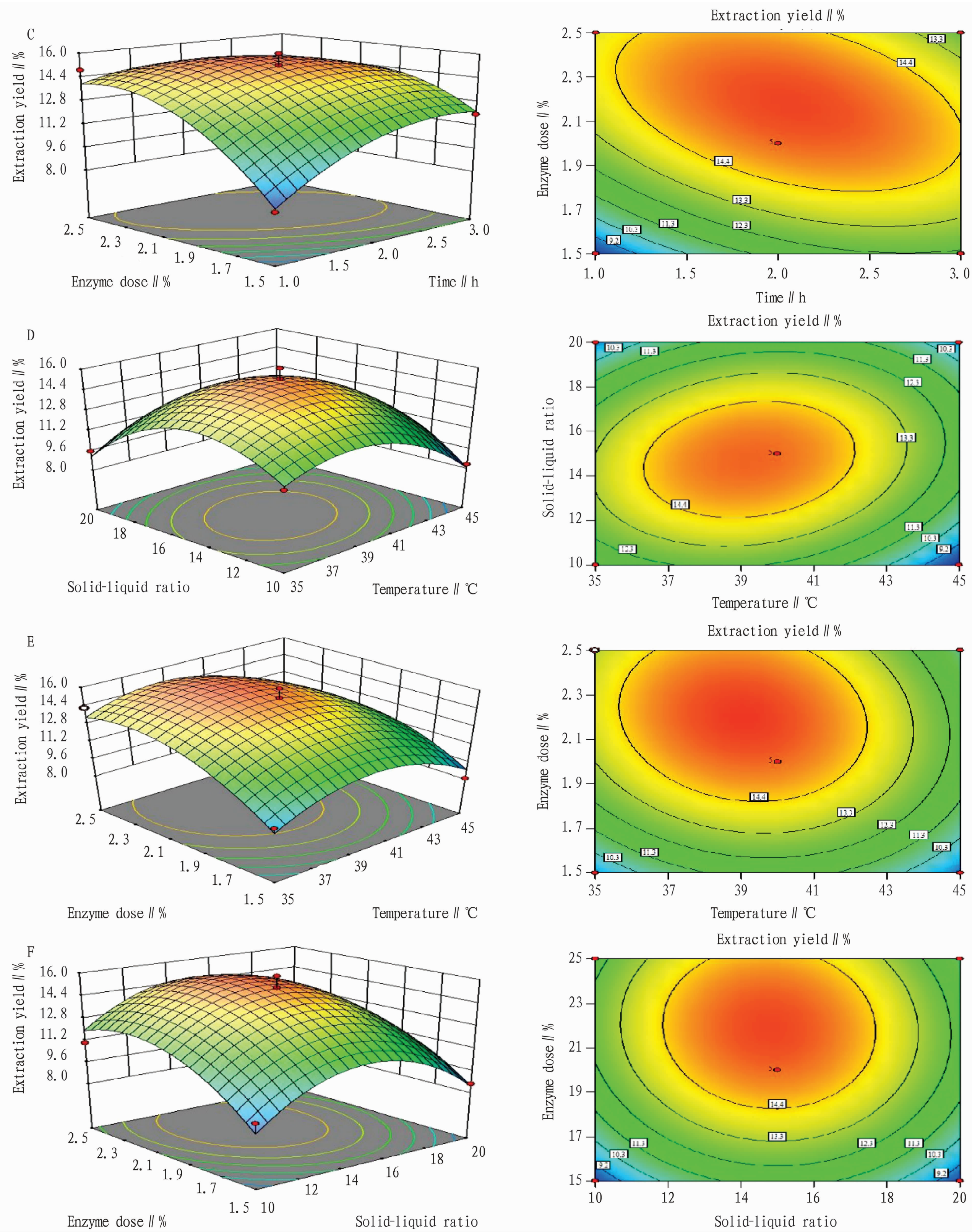
Source	Sum of squares	*df*	Mean square	*F* value	value Prob > *F*	Significance
Model	158.48	14	11.32	12.40	<0.000 1	* *
X_1-Time	4.95	1	4.95	5.42	0.035 4	*
X_2-Temperature	6.62	1	6.62	7.25	0.017 5	*
X_3-Solid-liquid ratio	0.45	1	0.45	0.50	0.492 5	
X_4-Enzyme dose	28.11	1	28.11	30.80	<0.000 1	* *
X_1X_2	6.74	1	6.74	7.39	0.016 7	*
X_1X_3	0.42	1	0.42	0.46	0.508 4	
X_1X_4	7.03	1	7.03	7.71	0.014 9	*
X_2X_3	3.07	1	3.07	3.36	0.088 2	
X_2X_4	1.26	1	1.26	1.38	0.258 9	
X_3X_4	0.22	1	0.22	0.24	0.628 8	
X_1^2	12.77	1	12.77	13.99	0.002 2	* *
X_2^2	42.08	1	42.08	46.11	<0.000 1	* *
X_3^2	64.34	1	64.34	70.50	<0.000 1	* *
X_4^2	26.92	1	26.92	29.49	<0.000 1	* *
Residual	12.78	14	0.91			
Lack of Fit	11.39	10	1.14	3.27	0.132 1	
Pure Error	1.39	4	0.35			
Cor Total	171.26	28				

*: Significant difference ($P<0.05$); * *: Extremely significant difference ($P<0.01$).

Model validation

The predicted optimal extraction conditions from software of Design Expert version 8.0.6 were as follows: extraction time 2 h, extraction temperature 40.55 ℃, solid-liquid ratio 1 : 15 (*w*/v), and enzyme dose 2.02%. The maximum response value predicted by the model was 14%. Considering the convenience and feasibility of the actual operation, the modified extraction conditions were as follows: extraction time 2 h, extraction temperature 40.5 ℃, solid-liquid ratio 1 : 15 (*w*/v), and enzyme dose 2.0%. Under these conditions, the average value of polysaccharide extraction yield of thrice experiments was (14.86 ± 0.12)%, which was in agreement with the theoretical value (15.89%) predicted by the model, suggesting the prediction result of the model was effective and reliable.





(A) Interaction effects between extraction temperature and time on extraction yield of polysaccharide; (B) Interaction effects between solid-liquid ratio and time on extraction yield of polysaccharide; (C) Interaction effects between enzyme dose and time on extraction yield of polysaccharide; (D) Interaction effects between solid-liquid ratio and temperature on extraction yield of polysaccharide; (E) Interaction effects between enzyme dose and temperature on extraction yield of polysaccharide; (F) Interaction effects between enzyme dose and solid-liquid ratio on extraction yield of polysaccharide.

Fig. 2 Interaction effects between various factors

Conclusions

In conclusion, the extraction process of MSP was optimized using single factor experiments and the response surface design. The optimal extraction conditions of MSP were as follows: extraction time 2 h, extraction temperature 40.5 ℃, solid-liquid ratio 1 : 15 (g/ml), and enzyme dose 2.02%. The extraction yield reached 15.89%.

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