Optimization of Ultrasonic-assisted Extraction Process for Polysaccharide from *Lactarius deliciosus* by Response Surface Methodology and Its Antioxidant Activity

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Abstract [**Objectives**] Polysaccharide was extracted from *Lactarius deliciosus* by ultrasonic-assisted method to improve polysaccharide yield. [**Methods**] Five variables including extraction temperature, extraction time, ultrasonic power, ultrasonic time and material-to-liquid ratio were selected for single factor experiments. The extraction process of *L. deliciosus* polysaccharide was optimized by response surface analysis, and its antioxidant activity was evaluated by measuring its total reduction ability and DPPH free radical scavenging capacity. [**Results**] The optimal extraction conditions were determined as follows: material-to-liquid ratio 1∶35 g∶ml, ultrasonic power 462 W, ultrasonic time 10 min, extraction time 110 min and extraction temperature 90 ℃. Under these conditions, the extraction rate of polysaccharide was (10.83 ±0.03)%. The antioxidant test results showed that when the mass concentration of *L. deliciosus* polysaccharide was 0.5 mg/ml, its absorbance and DPPH free radical scavenging rate reached their maximum values, which were 3.274% and 41.27%, respectively. The *L. deliciosus* polysaccharide had good antioxidant properties. [**Conclusions**] This study provides a theoretical basis for further development and utilization of *L. deliciosus* polysaccharide in the future.

Key words *Lactarius deliciosus*; Polysaccharide; Ultrasonic-assisted extraction; Response surface analysis; Antioxidant activity

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Lactarius deliciosus is an ectomycorrhizal edible fungus[1], belonging to *Lactarius*[2] of Russulaceae. It is widely planted and mainly distributed in the middle and lower reaches of the Yangtze River. *L. deliciosus* is delicious and deeply loved by the public. It has high medicinal and economic value[3], and is rich in dietary fiber[4], polysaccharides, crude protein, crude fat and eight kinds of essential amino acids[5]. Its polysaccharide is also commonly used in the research of immune cell activity, tumor resistance[6], blood sugar lowering and bacteriostasis[7]. The results showed that *L. deliciosus* polysaccharide has many activities, such as enhancing immune system function, inducing tumor cell apoptosis[8] and antioxidation[9]. Therefore, *L. deliciosus* polysaccharide has a good development prospect and scientific research significance.

At present, the extraction of polysaccharides from edible fungi mostly adopts water extraction and alcohol precipitation, alkali extraction, microwave-assisted method and ultrasonic-assisted method[10]. In view of the fact that water extraction and alcohol precipitation are common methods for the extraction of polysaccharide from *L. deliciosus*, there is no research report on ultrasonic-assisted extraction of polysaccharide from *L. deliciosus*. Cui[11] extracted the polysaccharide from *L. deliciosus* by hot water extraction, which was simple, but time-consuming and inefficient. Dong *et al.*[12] extracted polysaccharide from *L. deliciosus* by microwave-assisted method, which improved the extraction efficiency, but the microwave power and time should be strictly controlled. Ultrasonic-assisted extraction of polysaccharides has the advantages of simple and easy operation, mild conditions, short time consumption and high extraction efficiency, and is also widely used in polysaccharide extraction process. Therefore, in this study, ultrasonic-assisted extraction was applied to optimize the extraction method of *L. deliciosus* polysaccharide and improve the yield of polysaccharide. Meanwhile, the antioxidant activity of *L. deliciosus* polysaccharide was studied by measuring its total reducing ability and DPPH free radical scavenging rate.

Materials and Methods

Materials and reagents

L. deliciosus: Jishou City, Xiangxi, Hunan; concentrated sulfuric acid and ascorbic acid: Sinopharm Chemical Reagent Co., Ltd.; DPPH radical: Shanghai Yuanye Biotechnology Co., Ltd; anhydrous ferric chloride: Sinopharm Chemical Reagent Co., Ltd.; disodium hydrogen phosphate: Sinopharm Chemical Reagent Co., Ltd.

Instruments and equipment

Ultrasonic cell pulverizer: Ningbo Scientz Biotechnology Co., Ltd.; 7230G visible spectrophotometer: Shanghai Yoke Instrument Co., Ltd.; LGJ-12 vacuum freeze dryer: Beijing Songyuan Huaxing Technology Development Co., Ltd.; 85-2 digital display temperature control magnetic agitator: Jintan Dadi Automation Instrument Factory.

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Bei LIU (2002 -), female, P. R. China, devoted to research about extraction and functional characteristics of food macromolecules.

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Experimental methods

Extraction of polysaccharides from *L. deliciosus* Dried *L. deliciosus* was pulverized, and sieved with a 60-mesh sieve to obtain *L. deliciosus* powder, which was stored in a sealed state for later use. A certain amount of *L. deliciosus* powder was added in a 100 ml beaker, which was then added with a certain volume of distilled water. The beaker was put in a water bath pot for thermal insulation extraction. The extract was centrifuged (3 000 r/min, 30 min) to obtain supernatant, *i.e.*, a crude polysaccharide solution, which was diluted to constant volume. After proper dilution, the absorbance was determined by phenol-sulfuric acid method and the yield of polysaccharides was calculated.

Determination of extraction rate of polysaccharides from *L. deliciosus* The phenol-sulfuric acid method was applied to determine the total content of *L. deliciosus* polysaccharide[13]. A standard glucose solution was prepared, and different volumes of the standard glucose solution were pipetted into test tubes, respectively. Distilled water was added to each test tube to 2.0 ml, and phenol and concentrated sulfuric acid were then added. After shaking well and cooling for 30 min, the absorbance was measured at 490 nm with a spectrophotometer. *A* standard glucose curve was drawn with glucose concentration c (mg/ml) as the *x*-axis and absorbance value *A* as the *y*-axis, and the linear regression formula of the standard curve was further obtained: $A = 10.728c + 0.0857$, with linear correlation coefficient $R^2 = 0.9988$.

Sample determination: The supernatant obtained after centrifugation and filtration was diluted to a constant volume of 100.0 ml, and mixed thoroughly and evenly. Next, 1.0 ml was added into a 10 ml test tube, which was then added with 1.0 ml of 5% phenol and 5.0 ml of concentrated sulfuric acid. After shaking to mix evenly and cooling, the absorbance value was measured at 490 nm with a spectrophotometer. Then, the absorbance value was substituted into the linear regression equation of standard curve to obtain the polysaccharide concentration diluted to constant volume. Finally, the yield (%) of *L. deliciosus* polysaccharide was calculated according to following formula:

$$\text{Yield of } L.\ deliciosus \text{ polysaccharide} = \frac{c \times v \times n}{m} \times 100\% \quad (1)$$

In the formula, c is the concentration of diluted polysaccharide solution, mg/ml; v is the constant volume, ml; n is the dilution ratio; and m is the mass of *L. deliciosus* powder, g.

Single factor experiments

With the yield of *L. deliciosus* polysaccharide as the index, the effects of material-to-liquid ratio, ultrasonic power, ultrasonic time, extraction time and extraction temperature on the yield of *L. deliciosus* polysaccharide were investigated, and each group of experiment was repeated three times.

Effects of single factors on extraction rate of *L. deliciosus* polysaccharide Multiple portions of dry *L. deliciosus* powder were weighed, 1.0 g each, and distilled water was added according to different material-to-liquid ratios (1∶20, 1∶25, 1∶30, 1∶35, 1∶40, g∶ml) to prepare solutions for later use. Then, the material was extracted under the conditions of ultrasonic power of 390 W, ultrasonic time of 10 min, extraction temperature of 90 ℃ and extraction time of 120 min. Next, 30 ml of distilled water was added to *L. deliciosus* powder to prepare solutions for later use. Then, the material was extracted with ultrasonic time as the variable (5, 10, 15, 20, 25 min), under the conditions of ultrasonic power of 390 W, extraction temperature of 90 ℃ and extraction time of 120 min. Next, 30 ml of distilled water was added to *L. deliciosus* powder to prepare solutions for later use. Then, the material was extracted with ultrasonic power as the variable (130, 260, 390, 520, 650 W), under the conditions of ultrasonic time of 10 min, extraction temperature of 90 ℃ and extraction time of 120 min. Next, 30 ml of distilled water was added to *L. deliciosus* powder to prepare solutions for later use. Then, the material was extracted with extraction time as the variable (60, 90, 120, 150, 180 min), under the conditions of ultrasonic time of 10 min, ultrasonic power of 390 W and extraction temperature of 90 ℃. Next, 30 ml of distilled water was added to *L. deliciosus* powder to prepare solutions for later use. Then, the material was extracted with extraction temperature as the variable (80, 85, 90, 95, 100 ℃), under the conditions of ultrasonic power of 390 W, ultrasonic time of 10 min, and extraction time of 120 min. The absorbance of extracted *L. deliciosus* polysaccharide was measured at the wavelength of 490 nm by a spectrophotometer, and the extraction rate of polysaccharides was calculated. The effects of different material-to-liquid ratio, ultrasonic time, ultrasonic power, extraction time and extraction temperature on the yield of *L. deliciosus* polysaccharide were studied.

Optimization of extraction process by response surface methodology

According to the results of single factor experiments mentioned above, extraction temperature had little effect on the yield of *L. deliciosus* polysaccharide. With such four factors as material-to-liquid ratio, extraction time, ultrasonic time and ultrasonic power as independent variables and the yield of polysaccharide as the response value, an experimental scheme was designed by Box-Behnken method. The optimum technological conditions for extracting *L. deliciosus* polysaccharide were obtained. Factors and levels of the response surface experiment are shown in Table 1.

Table 1 Factors and levels in response surface experiment

Level	Factor			
	Material-to-liquid ratio (A)//g/ml	Extraction time (B)//min	Ultrasonic time (C)//min	Ultrasonic power (D)//W
−1	25	90	5	260
0	30	120	10	390
1	35	150	15	520

Determination of antioxidant activity of polysaccharides from *L. deliciosus*

Determination of total reducing capacity The total reducing ability of *L. deliciosus* polysaccharide was determined by the potassium ferricyanide reduction method of Zhao *et al.*[14].

Determination of DPPH radical scavenging rate The DPPH

radical scavenging rate of *L. deliciosus* polysaccharide was determined according to the method of Wang *et al.*[15].

Results and Analysis

Single factor experiments

Effects of material-to-liquid ratio on extraction rate of polysaccharides from *L. deliciosus* From Fig. 1A, it can be seen that when the ratio of material to liquid was in the range of 1∶20 to 1∶30 g∶ml, the yield of *L. deliciosus* polysaccharide increased with the ratio of material to liquid increasing, and when the ratio of material to liquid was 1∶30 g∶ml, the yield of *L. deliciosus* polysaccharide reached the maximum value of 10.04%. With the ratio of solid to liquid further increasing, the yield of polysaccharides from *L. deliciosus* decreased, but the trend was slow. The reason might be that with the increase of material-to-liquid ratio, it was helpful for the dissolution and diffusion of *L. deliciosus* polysaccharide, but when the material-to-liquid ratio increased to a certain extent, *L. deliciosus* polysaccharide had been fully dissolved in the solvent, or the dissolution of other water-soluble substances in the raw material affected the extraction of polysaccharide[16].

Effects of ultrasonic time on extraction rate of polysaccharide from *L. deliciosus* It can be seen from Fig. 1B that in a certain ultrasonic time range, with the ultrasonic time increasing, the yield of *L. deliciosus* polysaccharide first increased and then decreased. When the ultrasonic time was 15 min, the yield of *L. deliciosus* polysaccharide reached a maximum at 10.04%. The reason might be that in a certain ultrasonic time range, sufficient ultrasonic treatment was beneficial to the dissolution of polysaccharide from *L. deliciosus*, and if the ultrasonic time was too long, the diffusion of polysaccharide could reach a balanced state. Meanwhile, too-long ultrasonic time made other active components dissolve out of the cell wall of *L. deliciosus*, inhibited the dissolution of polysaccharide, and reduced the yield of *L. deliciosus* polysaccharide[17].

Effects of ultrasonic power on extraction rate of polysaccharide from *L. deliciosus* It can be seen from Fig. 1C that with ultrasonic power increasing in the range of 130 – 650 W, the yield of *L. deliciosus* polysaccharide first increased and then decreased. When the ultrasonic power was 390 W, the yield of *L. deliciosus* polysaccharide reached a maximum value of 9.65%. With the continuous increase of ultrasonic power, the yield of *L. deliciosus* polysaccharide decreased obviously. The reason might be that with the increase of ultrasonic power in a proper range, the cavitation effect generated by ultrasonic wave was enhanced, so that *L. deliciosus* polysaccharide could be dissolved better. However, with the further increase of ultrasonic power, a large number of tiny bubbles generated, which led to the decrease of cavitation effect[18], and then the yield of *L. deliciosus* polysaccharide decreased.

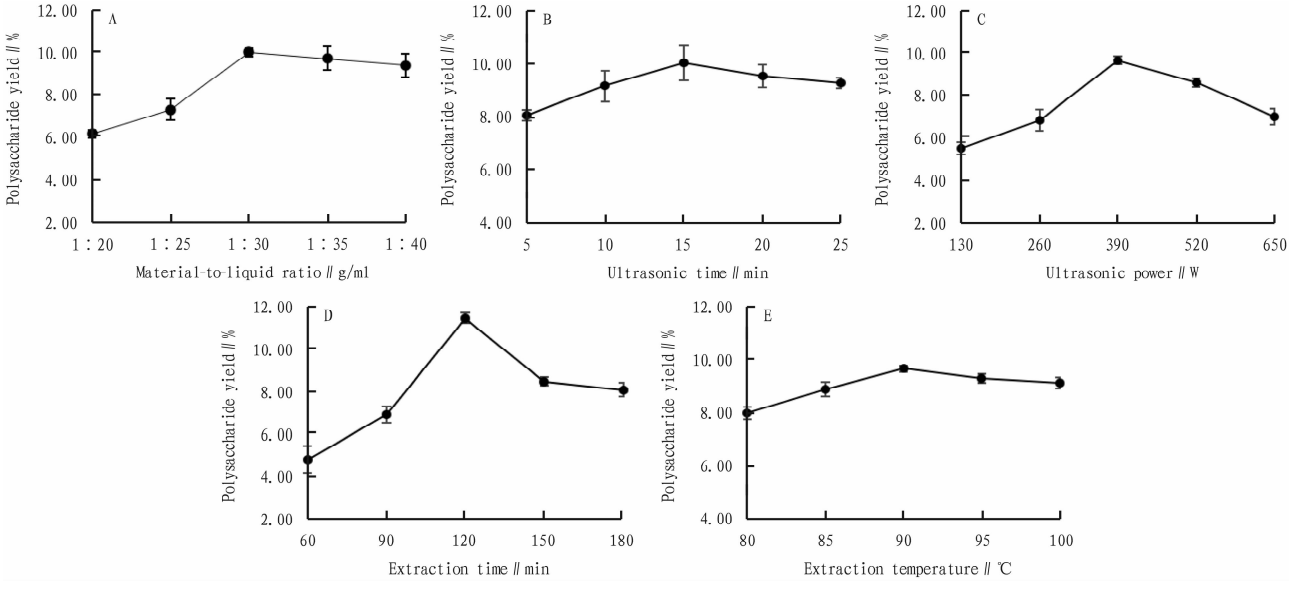

Fig. 1 Effects of material-to-liquid ratio (A), ultrasonic time (B), ultrasonic power (C), extraction time (D), and extraction temperature (E) on the extraction rate of *L. deliciosus* polysaccharide

Effects of extraction time on extraction rate of polysaccharides from *L. deliciosus* It can be seen from Fig. 1D that when the extraction time of *L. deliciosus* polysaccharide was within the range of 60 – 120 min, the yield of *L. deliciosus* polysaccharide showed a significant upward trend with the extraction time increasing. When the extraction time was 120 min, the yield of *L. deliciosus* polysaccharide reached a maximum at 9.44%. With the extraction time further increasing, the yield of *L. deliciosus* polysaccharide decreased significantly. The reason might be that the extraction temperature of *L. deliciosus* polysaccharide was relatively high, and long-term extraction led to denaturation and precipitation of glycoprotein in crude polysaccharide, and some polysaccharides were degraded, resulting in a decrease in the yield of *L. deliciosus* polysaccharide[19].

Effects of extraction temperature on extraction rate of polysaccharide from *L. deliciosus* From Fig. 1E, it can be seen that the extraction rate of *L. deliciosus* polysaccharide increased with the increase of water bath extraction temperature when the

water bath extraction temperature of *L. deliciosus* solution was between 80 and 90 ℃, and the extraction rate of *L. deliciosus* polysaccharide reached a maximum value of 9.67% when the extraction temperature reached 90 ℃. With the extraction temperature further increasing, the extraction rate of *L. deliciosus* polysaccharide showed a downward trend. The reason might be that increasing the extraction temperature speeded up the movement of solute molecules and solvent molecules, thus improving the yield of polysaccharide. When the temperature was too high, glycoprotein was easily degraded, and the vapor pressure increased at high temperature, and the cavitation buffering effect weakened the ultrasonic cavitation[20], which led to the decline of the yield of *L. deliciosus* polysaccharide.

Optimization of response surface experiment

Results of response surface optimization experiment Design-Expert 8.0.6.1 software was used to design the experiment. With the yield of *L. deliciosus* polysaccharide as the response value, four-factor three-level response surface analysis was conducted. The experimental design and results are shown in Table 2.

Regression model and variance analysis Design Expert 8.0.6.1 software was employed to perform multiple regression fitting on the experimental results. The quadratic multiple regression model equation for the extraction rate (Y) of *L. deliciosus* polysaccharide was obtained as follows:

$$Y = +10.10 + 1.15A + 0.43B + 5 \times 10^{-3} C + 1.38D - 0.82AB + 0.15AC + 1.72AD + 0.43BC - 0.15BD - 0.34CD - 1.57A^2 - 0.70B^2 - 0.60C^2 - 2.43D^2$$

The variance analysis of the model showed that the F value of the model was 16.75, which showed that the linear relationship between the dependent variable and the independent variables of the response surface model was obvious. The model $P < 0.000\ 1$ showed that the response surface regression model was at an extremely significant level, which could well reflect the relationship between the four factors and the extraction rate. The P value of the lack of fit term was not significant, which showed that the actual measured values could be well fitted with the regression model. The correlation coefficient $R^2 = 0.943\ 7$ indicated that the test error was small; and the adjusted correlation coefficient was calculated as $R_{Adj}^{\ 2} = 0.883\ 7$. To sum up, this model could be used to analyze the extraction rate of *L. deliciosus* polysaccharide. Meanwhile, in the regression equation, the effects of experimental factors could be judged according to the P value. The effects of A, D, AD, A^2 and D^2 on the extraction rate of *L. deliciosus* polysaccharide were extremely significantly ($P < 0.000\ 1$), and the effects of B, AB, B^2 and C^2 were significant ($P < 0.05$), while those of C, AC, BC, BD and CD were not significant. The effects of factors on the extraction rate of *L. deliciosus* polysaccharide ranked as D > A > B > C.

Response surface analysis The interaction between various elements is shown in Fig. 2, which can directly reflect the interaction between the four factors. Among them, the steep gradient of 3D surface could directly reflect the effects of two factors on the response value. The steeper the gradient, the greater the effects of the factor on the extraction rate of *L. deliciosus* polysaccharide. In Fig. 2a, the slope of the curved surface of A was steeper than that of B, so the factors affecting the extraction rate of *L. deliciosus* polysaccharide ranked as A > B. Similarly, following conclusions could be obtained from Fig. 2b, Fig. 2c, Fig. 2d and Fig. 2f: A > C, D > A, D > B, B > C and D > C. Therefore, the primary and secondary order of factors affecting the extraction rate of *L. deliciosus* polysaccharide was D > A > B > C, which was consistent with the analysis of variance.

Table 2 Box-Behnken test design and results

Experiment	Material-to-liquid ratio (A)//g/ml	Extraction time (B)//min	Ultrasonic time (C)//min	Ultrasonic power (D)//W	Extraction rate//%
1	35	120	10	520	9.75
2	25	150	10	390	9.02
3	30	150	10	520	8.06
4	30	120	10	390	10.02
5	30	150	15	390	9.13
6	35	120	5	390	9.19
7	35	150	10	390	9.07
8	25	120	10	260	5.76
9	30	120	10	390	10.06
10	35	150	10	390	8.61
11	30	120	10	390	10.01
12	30	90	5	390	9.21
13	25	120	10	520	4.27
14	30	90	10	520	8.04
15	35	120	15	390	9.63
16	25	120	15	390	6.14
17	30	120	15	260	5.68
18	30	90	15	390	8.13
19	30	120	15	520	8.91
20	30	120	10	390	10.18
21	30	150	10	260	5.97
22	25	90	10	390	5.29
23	30	90	10	260	5.34
24	25	120	5	390	6.31
25	35	120	10	260	4.38
26	30	120	10	390	10.21
27	30	120	5	260	4.88
28	30	150	5	390	8.49
29	30	120	5	520	9.48

Verification test Through the above analysis, the optimum extraction conditions of *L. deliciosus* polysaccharide were as follows: material-to-liquid ratio 1∶33.61 (g∶ml), ultrasonic power 462.08 W, ultrasonic time 9.3 min, and extraction time 113.4 min. Under these conditions, the theoretical extraction rate of *L. deliciosus* polysaccharide was 10.85%. Considering the operability of the experiment, the optimum ultrasonic-assisted extraction process of *L. deliciosus* polysaccharide was as follows: material-to-liquid ratio 1∶35 g∶ml, ultrasonic power 462 W, ultrasonic time 10 min, and extraction time 110 min. In the verification test, the optimal technical conditions were used for extraction, and the actual extraction rate of *L. deliciosus* polysaccharide was 10.83 ± 0.03%, which was close to the predicted result, indicating that the model was reliable.

Table 3 Analysis of variance of regression equation

Source of variation	Sum of squares	Degree of freedom	Mean square	*F*	*P*	Significance
Model	103.29	14	7.38	16.75	<0.000 1	* * *
A	15.96	1	15.96	36.24	<0.000 1	* * *
B	2.18	1	2.18	4.96	0.042 9	*
C	3.00×10^{-4}	1	3.00×10^{-4}	6.812×10^{-4}	0.979 5	
D	22.69	1	22.69	51.51	<0.000 1	* * *
AB	2.67	1	2.67	6.07	0.027 3	*
AC	0.093	1	0.093	0.21	0.652 9	
AD	11.76	1	11.76	26.71	0.000 1	* * *
BC	0.74	1	0.74	1.68	0.216 0	
BD	0.093	1	0.093	0.21	0.652 9	
CD	0.47	1	0.47	1.07	0.319 5	
A^2	15.95	1	15.95	36.21	<0.000 1	* * *
B^2	3.18	1	3.18	7.23	0.017 7	*
C^2	2.32	1	2.32	5.27	0.037 7	*
D^2	38.32	1	38.32	87.00	<0.000 1	* * *
Residual	6.17	14	0.44			
Lack of fit	6.13	10	0.61			
Pure error	0.035	4	8.633×10^{-3}	71.05	0.000 5	
Total	109.45	28				

* * * indicates an extremely significant difference ($P<0.000\ 1$); * * stands for a highly significant difference ($0.000\ 1<P<0.01$); and * indicates a significant difference ($P<0.05$).

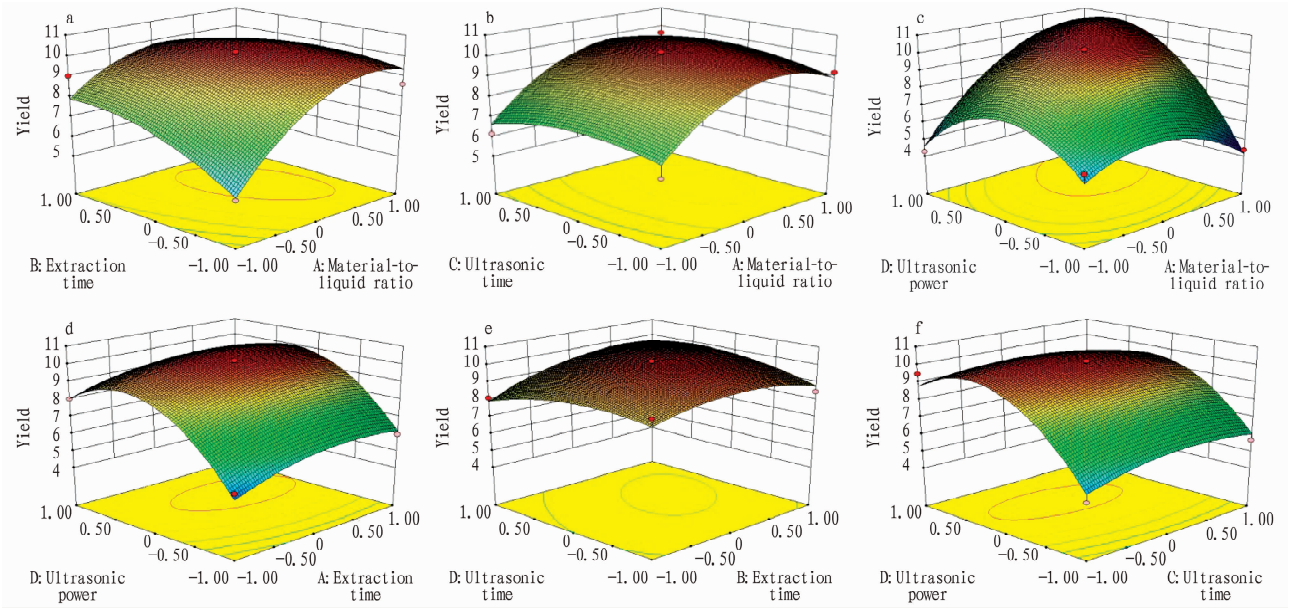

Fig. 2 Response surface plot of effects of interaction between various factors on the yield of *L. deliciosus* polysaccharide

Determination of antioxidant activity of polysaccharide from *L. deliciosus in vitro*

Total reduction capacity As shown in Fig. 3A, in the range of 0.1 – 0.5 mg/ml, the absorbance of *L. deliciosus* polysaccharide was significantly different from that of the V_C control group ($P<0.05$). The total reducing capacity of *L. deliciosus* polysaccharide was positively correlated with the mass concentration. When the mass concentration of *L. deliciosus* polysaccharide was increased, its total reducing capacity was gradually enhanced, and the absorbance of polysaccharide solution reached a maximum value of 0.331 when the mass concentration was 0.5 mg/ml. At this time, the total reducing capacity was the best. Compared with the total reducing capacity of V_C solution at the same concentration, the total antioxidant capacity of *L. deliciosus* polysaccharide was weaker.

DPPH radical scavenging ability As shown in Fig. 3B, in the range of 0.1 – 0.5 mg/ml, the scavenging rate of *L. deliciosus* polysaccharide on DPPH radicals was significantly different from that of the V_C control group ($P<0.05$). The greater the concentration of *L. deliciosus* polysaccharide, the stronger the scavenging ability of DPPH free radicals. When the mass concentration was 0.5 mg/ml, the DPPH free radical scavenging ability of *L. deliciosus* polysaccharide was the strongest, at 34.9%, indicating that the DPPH free radical scavenging ability of *L. deliciosus* polysaccharide solution was relatively strong, but weaker than that of V_C.

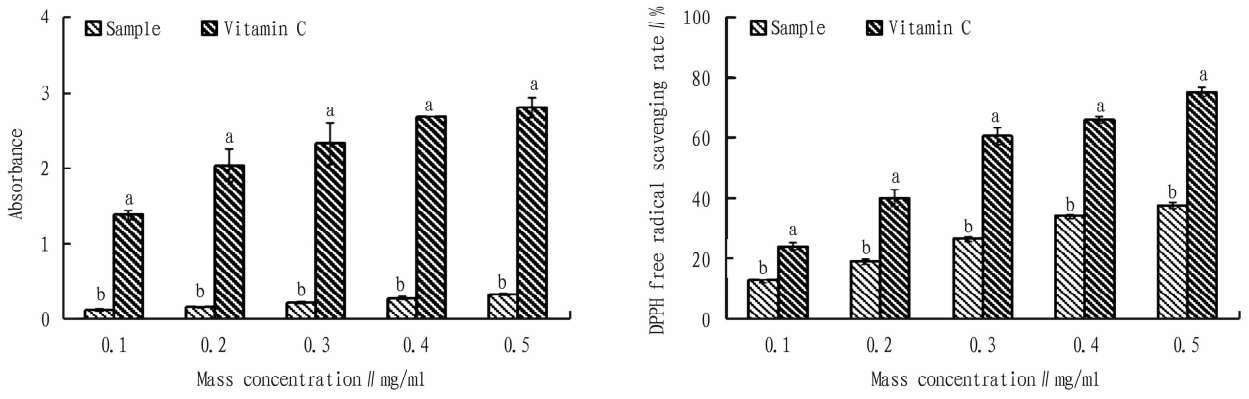

Fig. 3 Total reducing capacity (A) and DPPH free radical scavenging rate (B) of *L. deliciosus* polysaccharide

Conclusions and Discussion

In this study, *L. deliciosus* was used as the raw material to extract polysaccharide by the ultrasonic-assisted method. The optimum extraction conditions were determined as follows: material-to-liquid ratio 1 ∶ 35 g ∶ ml, ultrasonic power 462 W, ultrasonic time 10 min, extraction time 110 min and extraction temperature 90 ℃. Under these conditions, the yield of *L. deliciosus* polysaccharide was determined to be 10.83% ±0.03%. The total reducing ability and DPPH free radical scavenging ability of *L. deliciosus* polysaccharide were measured, and it was concluded that *L. deliciosus* polysaccharide had good antioxidant ability. This study provides better extraction conditions and methods for *L. deliciosus* polysaccharide, and lays a theoretical foundation for the application of *L. deliciosus* polysaccharide in beauty product and medical industries.

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