Intracellular Polysaccharide and Its Antioxidant Activity by *Cordyceps militaris* SU-08

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ABSTRACT: The extraction conditions of intracellular polysaccharide from *Cordyceps militaris* SU-08 mycelia in submerged culture were investigated. Four parameters affecting the IPS extraction were determined by Plackett-Burman (PB) tests and then optimized by response surface methodology (RSM). The optimum conditions of IPS extraction were predicted to be, ultrasonic treatment number 61.45, ultrasonic power 543.64 W, ethanol multiple 3.28, and extraction temperature 82.61°C, and the extraction rate of IPS was estimated at 9.11%. The actual value of IPS under these conditions was 9.19%. The *in vitro* antioxidant results showed that the inhibition effects of IPS at a dosage of 5 g L^{-1} on hydroxyl, superoxide anion, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical were 51.89% ± 3.27%, 82.26% ± 5.03%, and 74.59% ± 4.53%, respectively, which were 32.98% ± 2.71%, 69.71% ± 4.24%, and 41.64% ± 3.28% higher than that of control, respectively. The reducing power of IPS was 0.79 ± 0.03 (absorbance at 700 nm), 11.27% ± 0.82% higher than that of control. The results provide a reference for large-scale extraction of IPS by *C. militaris* SU-08 in industrial fermentation and the IPS can be used as a potential antioxidant which enhances adaptive immune responses. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 120: 1744–1751, 2011

Key words: antioxidants; polysaccharides; biopolymers

INTRODUCTION

Cordyceps militaris, a traditional medicine in China for treatment of a variety of diseases, contains many biological bioactive materials, such as cordycepin, cordycepic acid, protein, trace elements, ash, and carbohydrates.[1–3] *C. militaris* has the functions of immune response, reducing blood pressure, antiphlogosis, antibacterium, and antiarrhythmia.[4,5] Polysaccharides from the fruiting bodies of *C. militaris* have potential antioxidation, antitumor, antivirus, and immunomodulating properties.[5,6]

Many reports concerning to the polysaccharide of mushrooms are mainly focused on the exopolysaccharide (EPS) of fermentation broth and polysaccharide of fruiting bodies by *Pleurotus sajor-caju*,[7] *P. nebrodensis*,[8] *Morchella esculenta*,[9] *C. militaris*,[10] *Tremella fuciformis*,[11] *C. jiangxiensis*,[12] *T. mesenterica*,[13] and *Grifola frondosa*.[14] Although the intracellular polysaccharide (IPS) extracted from the mycelia of *P. ferulae*,[15] *Hypsizigus marmoreus*,[16] and *Marasmius androsaceus*[17] have been reported, the IPS of *C. militaris* and its antioxidant activities *in vitro* have not been studied.

The objectives of this study were to optimize the extraction parameters for *C. militaris* SU-08 IPS produced during submerged culture by Plackett-Burman (PB) tests and response surface methodology (RSM), and to evaluate the *in vitro* antioxidant activities of IPS with the hydroxyl, superoxide anion, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and reducing power as main index.

EXPERIMENTAL

Chemicals

Butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), methionine (MET), DPPH and riboflavin (RF) were from Sigma Chemicals (St. Louis, USA). All other chemicals used in this experiment were analytical reagent grade and purchased from local chemical suppliers in China.

Microorganism and liquid culture

C. militaris SU-08 was provided by our laboratory and maintained on synthetic potato dextrose agar (PDA). The cultures were incubated for 7 days at 25°C, stored at 4°C and subcultured every 3 months. Cultivation in liquid media was carried out in 250-mL Erlenmeyer flasks containing 100 mL of (g L^{-1}): potato, 200; glucose, 20; KH_2PO_4, 1.5, and $MgSO_4 \cdot 7H_2O$,

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TABLE I
Levels and Codes of Variables for Plackett–Burman Design

Variables	Symbol code	Coded levers		
		−1	0	1
Ultrasonic treatment time (s)	A_1	400	600	800
Ultrasonic treatment number[a]	A_2	40	60	80
pH	A_3	5	7	9
Ultrasonic power (W)	A_4	400	600	800
Water multiple	A_5	20	30	40
Ethanol multiple	A_6	1	2.5	4
Extraction time (h)	A_7	1	2	3
Extraction temperature (°C)	A_8	50	70	90
Precipitation time (h)	A_9	8	16	24
Precipitation temperature (°C)	A_{10}	−4	4	12

[a] 10 s each time at an interval 10 s.

1 with natural pH. Flasks were inoculated with a 0.5-cm^2 mycelial block of *C. militaris* SU-08 from the solid media, incubated at 25°C for 24 h without shaking, and then shaken on a rotary shaker (Anting, Shanghai, China) at 160 rpm for 5 days.

Measurement and preparation of IPS

The mycelia precipitates of *C. militaris* SU-08 were obtained by centrifugation at 3000 × *g* for 15 min and the cell wall of mycelium was broken by ultrasonic processor (Bingyang, Beijing, China) at 500 W for 10 min. After centrifugation (3000 × *g*, 15 min), protein was removed from the prepared supernatant by the method of Sevag.[18] The supernatant liquid was mixed with 3 vol. of 95% ethanol (v/v), stirred vigorously and kept at 4°C for 24 h. The precipitated IPS was dissolved in distilled water (60°C), and the IPS content was determined by the phenol-sulfuric acid method, using glucose as the standard.[19] IPS powder was obtained by quick prefreezing at −35°C for 1 h and then by vacuum freeze drying (Labconco, USA) for 6 h, and applied to evaluate the antioxidant activities *in vitro*.

PB experiments for IPS extraction

Initial screening of the most significant parameters affecting IPS extraction by *C. militaris* SU-08 was performed by PB design as reported by Plackett and Burman.[20] Ten variables including ultrasonic treatment time, ultrasonic treatment number, ultrasonic power, water multiple, ethanol multiple, extraction time, extraction temperature, precipitation time, precipitation temperature and pH were investigated in this experiment. In addition, five center points were added for the variables that could be assigned numerical values. The extraction rate of IPS was expressed as a percentage of IPS to mycelium (w/w). The experimental design with the name, symbol code, and actual level of the variables is shown in Tables I and II.

Response surface optimization for IPS extraction

Based on the results of the PB tests, ultrasonic treatment number, ultrasonic power, ethanol multiple, and extraction temperature were chosen for optimization of IPS extraction by the Box-Behnken design. The experimental design with name, symbol code, and actual level of the variables is shown in Tables III and IV. The test factors were coded according to the following equation:

$$x_i = (X_i - X_0)/\Delta X_i \quad i = 1, 2, 3, \ldots, k \tag{1}$$

where x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of the independent variable at the center point, and ΔX_i is the step change value.

To correlate the response variable to the independent variables, the following quadratic polynomial equation was applied to fit the response variable to a quadratic model:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \tag{2}$$

where Y is the predicted response value, β_0 is the intercept term, β_i is the linear term, β_{ii} is the squared term, β_{ij} is the interaction term, x_i and x_j are the coded level of independent variables.

TABLE II
Results of Plackett–Burman for IPS Extraction by *C. militaris* SU5–08

Runs	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	A_{10}	IPS yield (%)
1	1	1	−1	1	1	1	−1	−1	−1	1	6.94
2	−1	1	1	−1	1	1	1	−1	−1	−1	6.15
3	1	−1	1	1	−1	1	1	1	−1	−1	7.67
4	−1	1	−1	1	1	−1	1	1	1	−1	7.63
5	−1	−1	1	−1	1	1	−1	1	1	1	7.27
6	−1	−1	−1	1	−1	1	1	−1	1	1	6.36
7	1	−1	−1	−1	1	−1	1	1	−1	1	5.21
8	1	1	−1	−1	−1	1	−1	1	1	−1	6.87
9	1	1	1	−1	−1	−1	1	−1	1	1	4.89
10	−1	1	1	1	−1	−1	−1	1	−1	1	6.89
11	1	−1	1	1	1	−1	−1	−1	1	−1	5.93
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	3.27
13	0	0	0	0	0	0	0	0	0	0	5.52
14	0	0	0	0	0	0	0	0	0	0	6.19
15	0	0	0	0	0	0	0	0	0	0	5.61
16	0	0	0	0	0	0	0	0	0	0	5.99
17	0	0	0	0	0	0	0	0	0	0	6.01
Significant		[a]	[b]	[a]		[a]		[a]			

[a] Significant at 1% level.
[b] Significant at 5% level.

TABLE III
Levels and Codes of Variables for Box–Behnken Design

Variables	Symbol		Coded levels		
	Uncoded	Coded	−1	0	1
Ultrasonic treatment number	X_1	x_1	40	60	80
Ultrasonic power (W)	X_2	x_2	400	600	800
Ethanol multiple	X_3	x_3	1	2.5	4
Extraction temperature (°C)	X_4	x_4	50	70	90

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was measured according to the method of Winterbourn and Sutton.[21] The reaction mixture contained 1 mL of 0.15 M phosphate buffer saline (pH 7.4), 1 mL of 40 μg mL^{-1} safranin, 1 mL of 0.945 m*M* EDTA-Fe (II), 1 mL of 3% (v/v) H_2O_2, and 0.5 mL of the IPS (0.05–5 g L^{-1}). After incubating at 37°C for 30 min, the absorbance of IPS was measured at 560 nm, using BHT as a positive control. The hydroxyl radical scavenging activity was expressed as:

$$\text{Scavenging rate } (\%) = [(A_0 - A_1)/A_0] \times 100\% \quad (3)$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of IPS/BHT.

Superoxide radical scavenging assay

Superoxide anion radical scavenging activity was determined according to method of Stewar and Beewley.[22] The reaction mixture (3 mL) contained 13 m*M* MET, 10 m*M* RF, 75 μ*M* NBT, 100 m*M* EDTA, 50 m*M* phosphate buffer (pH 7.8), and the IPS (0.05–5 g L^{-1}). After illuminating the reaction mixture with a fluorescent lamp at 25°C for 30 min, the absorbance of the IPS was measured at 560 nm, using BHT as a positive control. The whole reaction was assembled in a box lined with aluminum foil. The scavenging rate was calculated using the following formula:

$$\text{Scavenging rate } (\%) = [(A_0 - A_1)/A_0] \times 100 \quad (4)$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of the IPS/BHT.

DPPH scavenging assay

The DPPH scavenging activity of IPS was measured according to the method of Liu and Zhao.[23] The reaction mixture contained 2 mL of 95% ethanol, 0.1 μ*M* DPPH, and 2 mL of the IPS (0.05–5 g L^{-1}). The solution was incubated at 25°C for 15 min, and the absorbance of IPS was determined at 517 nm using BHT as a positive control. The antioxidant activity of IPS was evaluated according to the following formula:

$$\text{Scavenging rate } (\%) = (1 - A/A_0) \times 100\% \quad (5)$$

where A was absorbance of IPS/BHT and A_0 was the absorbance of the DPPH solution.

Determination of reducing power

The reducing power of IPS was evaluated according to the method of Oyaizu[24] with slight modification. The reaction mixtures contained 2.5 mL phosphate buffer (pH 6.6, 0.2 M), 2.5 mL potassium ferricyanide (1%, w/v), and the IPS (0.05–5 g L^{-1}). After incubating at 50°C for 20 min, 2.5 mL of trichloroacetic acid (10%, w/v) was added to the mixture for terminating the reaction, and then centrifuged at 1200 × *g* for 10 min. An aliquot of 2.5 mL supernatant was collected and mixed with 2.5 mL deionized water and 0.5 mL $FeCl_3$ (0.1%, w/v). After incubating at room temperature for 15 min, the absorbance of the IPS was measured at 700 nm, using BHT as a positive control.

TABLE IV
Experimental and Predicted Values of IPS Based on Box−Behnken Design

Runs	x_1	x_2	x_3	x_4	IPS yield (%)	
					Experimental	Predicted
1	−1	−1	0	0	4.79	4.91
2	1	−1	0	0	4.85	5.31
3	−1	1	0	0	5.51	5.55
4	1	1	0	0	6.54	6.91
5	0	0	−1	−1	3.28	3.52
6	0	0	1	−1	6.49	6.2
7	0	0	−1	1	4.21	4.98
8	0	0	1	1	8.79	8.52
9	−1	0	0	−1	4.83	5.01
10	1	0	0	−1	5.2	5.22
11	−1	0	0	1	6.41	6.48
12	1	0	0	1	8.09	8.02
13	0	−1	−1	0	3.99	3.28
14	0	1	−1	0	4.55	4.21
15	0	−1	1	0	5.99	6.44
16	0	1	1	0	6.97	7.76
17	−1	0	−1	0	3.09	3.18
18	1	0	−1	0	4.68	4.56
19	−1	0	1	0	7.54	7.04
20	1	0	1	0	8.12	7.42
21	0	−1	0	−1	4.89	4.95
22	0	1	0	−1	5.24	5.01
23	0	−1	0	1	6.42	6.03
24	0	1	0	1	8.89	8.21
25	0	0	0	0	8.67	8.7
26	0	0	0	0	8.83	8.7
27	0	0	0	0	8.82	8.7
28	0	0	0	0	8.52	8.7
29	0	0	0	0	8.68	8.7

TABLE V
ANOVA for the Evaluation of the Quadratic Model

Source	Coefficients	S.E.	Sum of squares	Mean square	*F*-value	*P*
Model	–	–	92.7636	6.6260	22.3032	<0.0001**
Intercept	8.70	0.2438	–	–	–	–
x_1 (number)	0.44	0.1573	2.3497	2.3497	7.9091	0.0138*
x_2 (power)	0.56	0.1573	3.8194	3.8194	12.8562	0.0030**
x_3 (multiple)	1.68	0.1573	33.7010	33.7010	113.4384	<0.0001**
x_4 (temperature)	1.07	0.1573	13.8031	13.8031	46.4615	<0.0001**
x_1x_2	0.24	0.2726	0.2352	0.2352	0.7918	0.3886
x_1x_3	−0.25	0.2726	0.2550	0.2550	0.8584	0.3699
x_1x_4	0.33	0.2726	0.4290	0.42905	1.4441	0.2494
x_2x_3	0.11	0.2726	0.0441	0.0441	0.1484	0.7058
x_2x_4	0.53	0.2726	1.1236	1.1236	3.7821	0.0722
x_3x_4	0.35	0.2726	0.4761	0.4761	1.6026	0.2262
x_1^2	−1.45	0.2140	13.6698	13.6698	46.0130	<0.0001**
x_2^2	−1.58	0.2140	16.1765	16.1765	54.4504	<0.0001**
x_3^2	−1.70	0.2140	18.6732	18.6732	62.8546	<0.0001**
x_4^2	−1.07	0.2140	7.3806	7.3806	24.8434	0.0002**
Lack-of-fit			4.0913	0.4091	24.1283	0.0038

$R^2 = 0.9571$.
$R = 0.9783$.
Adj-$R^2 = 0.9142$.
* Significant at 5% level.
** Significant at 1% level.

Statistical analysis

All experiments were carried out in triplicates. Data were processed and analyzed using Design Expert Software (version 7.1.3, Stat-Ease, Minneapolis, MN) including ANOVA.

RESULTS AND DISCUSSION

Determination of parameters of IPS extraction

When the optimal extraction parameters were ultrasonic treatment time 800 s, ultrasonic treatment number 40, pH 9, ultrasonic power 800 W, water multiple 20, ethanol multiple 4, extraction time 3 h, extraction temperature 90°C, precipitation time 8 h, and precipitation temperature −4°C, the maximum rate of IPS extraction reached 7.67% (Table II). ANOVA results showed that ultrasonic treatment number, ultrasonic power, ethanol multiple, and extraction temperature had a highly significant influence on IPS extraction at the 1% level, and the influence of other parameters was at the 5% level or not significant ($P > 0.05$). Therefore, these four factors were chosen to optimize the process of IPS extraction using RSM.

Response surface optimization of IPS extraction

The experiments were planned to obtain a quadratic model consisting of 24 runs and five center points. The range and levels of three independent variables are shown in Table III. The Box-Behnken design matrix together with the experimental and predicted IPS data is shown in Table IV, while adequacy and fitness were evaluated by ANOVA (Table V).

By using multiple regression analysis, the polynomial model for an empirical relationship between the extraction rate of IPS and test variables in coded units was expressed by eq. (6).

$$\begin{aligned} Y_{\mathrm{IPS}} = {} & 8.7 + 0.44x_1 + 0.56x_2 + 1.68x_3 + 1.07x_4 \\ & + 0.24x_1x_2 - 0.25x_1x_3 + 0.33x_1x_4 + 0.11x_2x_3 \\ & + 0.53x_2x_4 + 0.35x_3x_4 - 1.45{x_1}^2 - 1.58{x_2}^2 \\ & - 1.7{x_3}^2 - 1.07{x_4}^2 \end{aligned} \quad (6)$$

where Y_{IPS} is the predicted response for IPS yield (%), and x_1, x_2, x_3, and x_4 are the coded test variables for ultrasonic treatment number, ultrasonic power (W), ethanol multiple, and extraction temperature (°C), respectively.

It can be seen from Table V that the linear term regression coefficients (x_1, x_2, x_3, x_4) and the quadratic coefficients (x_1^2, x_2^2, x_3^2, x_4^2) were significant at the 1 or 5% level, indicating that the ultrasonic treatment number, ultrasonic power, ethanol multiple, and extraction temperature are all significantly correlated with the yield of IPS extraction. The model was also significant ($P < 0.0001$) with a very high *F*-value (22.3032). The value of correlation coefficient ($R = 0.9783$) indicated good agreement between the experimental and predicted values of IPS, and R^2 (determinations coefficient) was 0.9571, showing a good agreement between experimental and

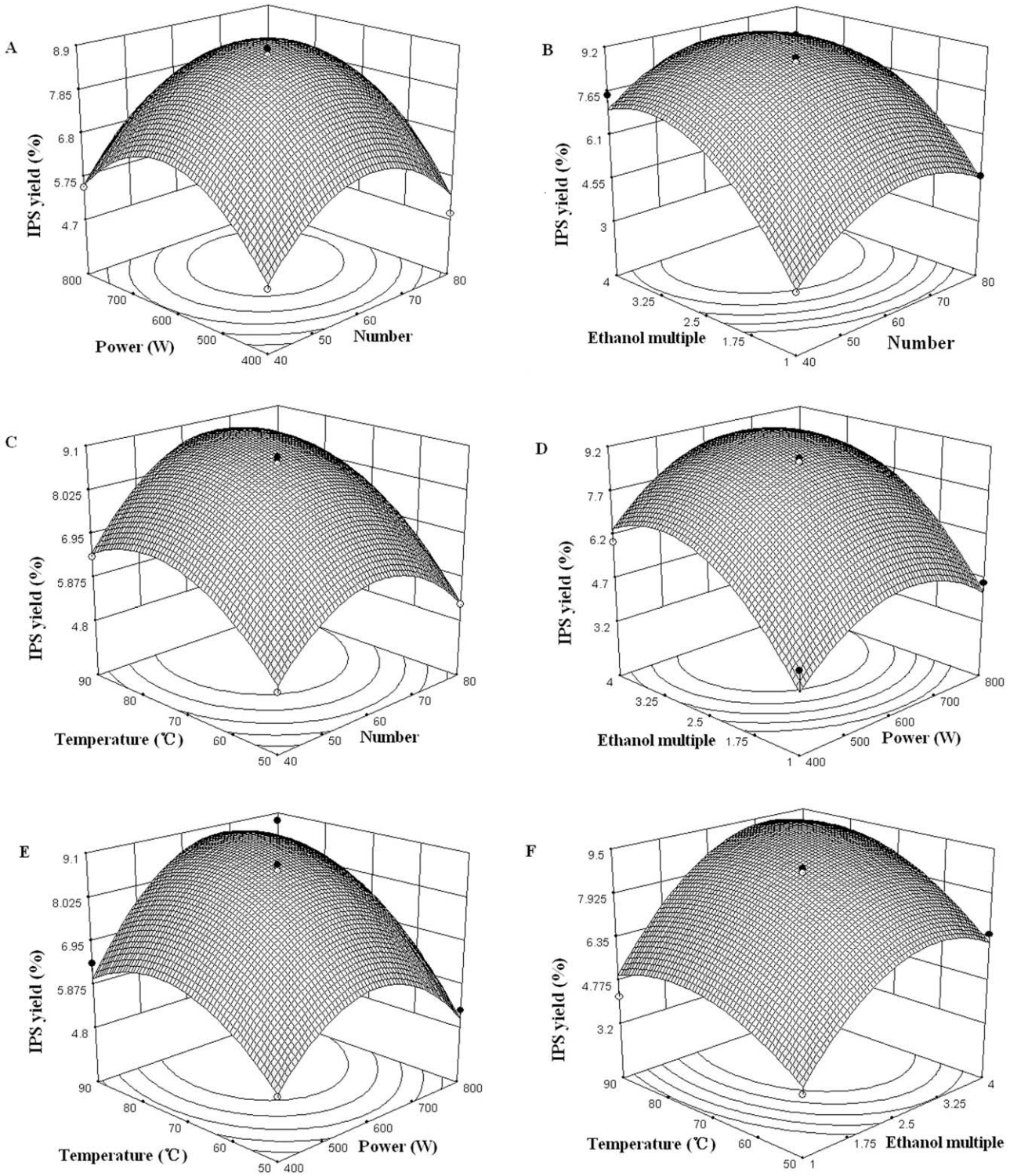

Figure 1 Response surface plot for the extraction yield of IPS of *C. militaris* SU-08 in terms of the effects of (A) power and number, (B) ethanol multiple and number, (C) temperature and number, (D) ethanol multiple and power, (E) temperature and power, and (F) temperature and ethanol multiple. Factors that were not included in the axes were fixed at their respective optimum levels.

predicted values which can explain 95.71% variability of the responses. The value of adjusted determinant coefficient (adj-R^2) was 0.9142, suggesting that the total variation of 91.42% for IPS is attributed to the independent variables and only nearly 9% of the total variation cannot be explained by the model. The *F*-value (24.1283) and *P*-value (0.0038) of lack-of-fit implied that it was not significant relative to the

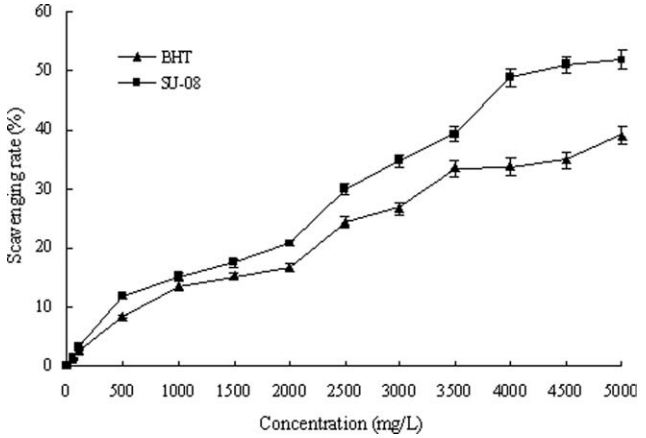

Figure 2 Scavenging effect of IPS on hydroxyl radical *in vitro*.

pure error, which indicated that the model equation was appropriate to predict the yield of IPS extraction under any combination of values.

To determine optimal levels of the test variables for IPS yield, the 3D response surface described by the regression model is presented in Figure 1. By solving the inverse matrix [from eq. (6)], the optimal values of the variables affecting IPS yield were: ultrasonic treatment number 61.45, ultrasonic power 543.64 W, ethanol multiple 3.28, and extraction temperature 82.61°C. Under these optimum conditions, the model gave the maximum predicted values of IPS extraction (9.11%), slightly lower than that obtained from the plot analysis (9.39%). In view of the operating convenience, the optimal extraction parameters were determined to be ultrasonic treatment number 60, ultrasonic power 545 W, ethanol multiple 3.3, and extraction temperature 80°C, while the predicted value of IPS extraction was 9.23%.

Triplicate experiments were performed under the determined conditions and the value of IPS extraction (9.19%) in agreement with the predicted value (9.23%) was obtained, which was much higher than

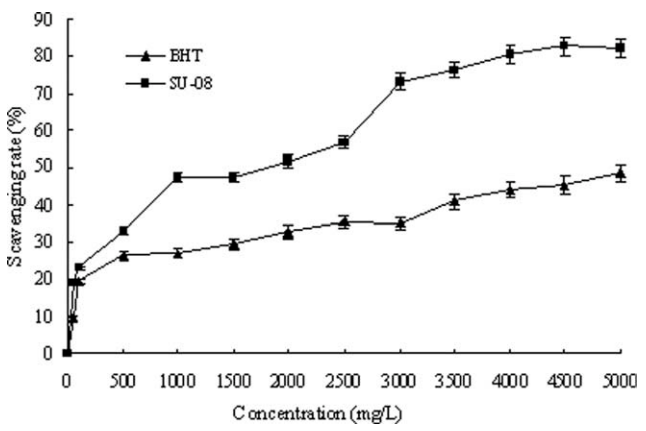

Figure 3 Scavenging effect of IPS on superoxide anion radical *in vitro*.

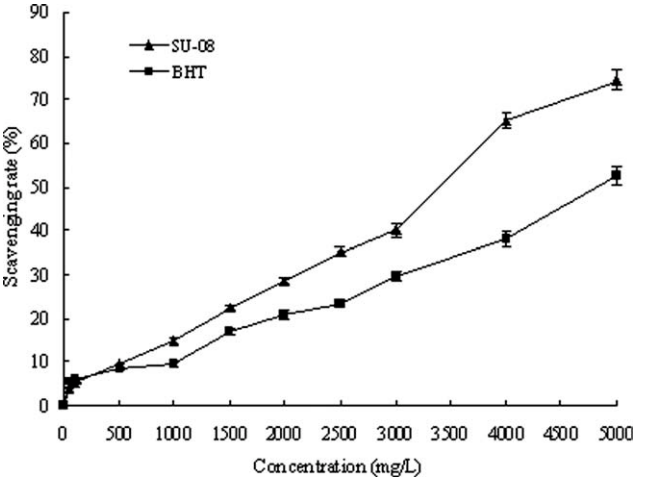

Figure 4 Scavenging effect of IPS on DPPH *in vitro*.

8.11% of *P. ferulae*,[15] 5.23% of *H. marmoreus*,[16] 8.57% of *M. androsaceus*,[17] and 5.78% of *C. militaris* fruit body,[25] respectively. The results indicated that the model was adequate for IPS extraction process.

Evaluation of antioxidant activities of IPS *in vitro*

Antioxidant activities have been attributed to various reactions and mechanisms, such as radical scavenging, reductive capacity, prevention of chain initiation, binding of transition metal ion catalysts, etc.[26] In this experiment, the *in vitro* antioxidant capacities of IPS were evaluated using different biochemical methods of hydroxyl, superoxide anion, DPPH radical scavenging assay, and reducing power analysis.

The hydroxyl radical scavenging results of IPS are described in Figure 2 and the inhibition activities of IPS and BHT were concentration-dependent at the dosage of 0.05–5 g L^{-1}. The scavenging percentage of IPS at 5 g L^{-1} reached 51.89% ± 3.27% ($P < 0.01$), which was 32.98% ± 2.71% higher than that of BHT (39.02% ± 2.16%, $P < 0.01$). It was also higher 45.7% of *C. sinensis*, [27] 25.5% of *Coprinus comatus*,[28] 24.8% of *T. fuciformis*,[29] 46.3% of *Coriolus versicolor*,[30] 26.2% of *Antrodia camphorate*,[31] 43.6% of *H. marmoreus*,[32] and 44.2% of *C. militaris* fruiting body,[33] respectively. The EC_{50} value of IPS was 4.26 ± 0.22 g L^{-1} ($P < 0.01$), which was lower than 5.1 g L^{-1} of *C. sinensis*,[27] 4.8 g L^{-1} of *C. versicolor*,[30] 5.1 g L^{-1} of *A. camphorate*,[31] 5.7 g L^{-1} of *H. marmoreus*,[32] and 6.4 g L^{-1} of *C. militaris* fruiting body,[33] respectively, indicating that the IPS of *C. militaris* SU-08 significantly affects the scavenging of hydroxyl radical.

Superoxide anion is one of the precursors of the singlet oxygen and hydroxyl radicals, therefore, it indirectly initiates lipid peroxidation. Apart from that, the presence of superoxide anion can magnify cellular damage because it produces other kinds of

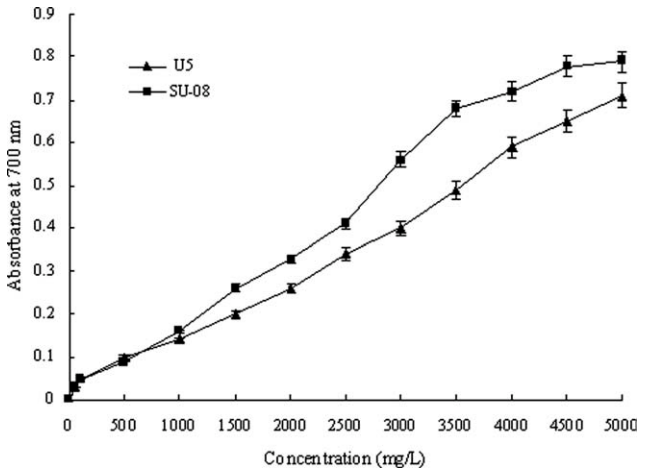

Figure 5 Reducing power of IPS.

free radicals and oxidizing agents.[34] The results of superoxide anion radical scavenging assay are shown in Figure 3. The scavenging rate of IPS at 5 g L^{-1} was 82.26% ± 5.03% ($P < 0.01$), 69.71% ± 4.24% higher than that of BHT (48.47% ± 3.11%, $P < 0.05$), which was much higher than 58.2% of *C. sinensis*,[27] 23.1% of *C. comatus*,[28] 17.7% of *T. fuciformis*,[29] 31.4% of *C. versicolor*,[30] 35.5% of *A. camphorate*,[31] and 32.3% of *H. marmoreus*,[32] respectively. Some reports showed that the EC_{50} values of IPS of *C. sinensis*,[27] *C. versicolor*,[30] and *A. camphorate*[31] were 6.5, 9.3, and 3.9 g L^{-1}, respectively, remarkably lower than that of IPS (1.86 ± 0.08 g L^{-1}, $P < 0.05$) in this experiment. These data showed that the IPS of *C. militaris* SU-08 can effectively protect cell from damage and lipid peroxidation.

DPPH is a stable free radical that shows maximum absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance such as an antioxidant, the radical would be scavenged and the absorbance is reduced.[35] As shown in Figure 4, the DPPH scavenging rate of IPS at a dosage of 5 g L^{-1} was 74.59% ± 4.53% ($P < 0.01$), which was not only 41.64% ± 3.28% higher than that of BHT (52.66% ± 3.85%, $P < 0.05$), but also higher than 62.4% of *C. sinensis*,[27] 61.7% of *C. versicolor*,[30] 55.8% of *H. marmoreus*,[32] 63.2% of *C. comatus*,[36] 55.4% of *Lentinus edodes*,[37] and 38.1% of *Volvariella volvacea*,[37] respectively. The EC_{50} value of IPS was 3.63 ± 0.22 g L^{-1} ($P < 0.01$), which was lower than 4.81 ± 0.34 g L^{-1} ($P < 0.05$) of BHT, 5.2 g L^{-1} of *C. versicolor*,[30] 4.4 g L^{-1} of *H. marmoreus*,[32] 3.8 g L^{-1} of *C. comatus*,[36] and 7.8 g L^{-1} of *C. militaris* fruiting body,[33] respectively. The DPPH scavenging results revealed that the IPS probably contained substances that were proton donors and could react with free radicals to convert them to stable diamagnetic molecules.

Figure 5 showed that the reducing power (absorbance at 700 nm) of IPS at 5 g L^{-1} was 0.79 ± 0.03 ($P < 0.01$), which was 11.27% ± 0.82% higher than that of BHT (0.71 ± 0.03, $P < 0.05$). It was also much higher than 0.53 of *C. sinensis*,[27] 0.42 of *C. comatus*,[36] 0.48 of *C. versicolor*,[30] 0.59 of *H. marmoreus*,[32] and 0.12 of *C. militaris* fruiting body,[33] respectively. These results indicated that the IPS of *C. militaris* SU-08 in this study has potential antioxidant capacities.

CONCLUSIONS

No reports are so far available in the literature regarding the optimization of IPS extraction by *C. militaris* SU-08 in submerged culture and its antioxidant activities *in vitro*. In this study, process parameters such as ultrasonic treatment number, ultrasonic power, ethanol multiple, and extraction temperature were systematically investigated for IPS extraction. Response surface methodology using second-order regression for a four-factor-three-level Box-Behnken design was a successful tool for extraction optimization of IPS by *C. militaris* SU-08 in submerged culture. The IPS showed antioxidant activities i*n vitro*. The results provide a reference for large-scale extraction of IPS by *C. militaris* SU-08 in industrial fermentation and the IPS can be used as a potential antioxidant which enhances adaptive immune responses.

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