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Full Length Research Paper

Extraction and *in vitro* antioxidant activity of exopolysaccharide by *Pholiota adiposa* SX-01

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The extraction parameters for *Pholiota adiposa* SX-01 exopolysaccharide (EPS) produced during submerged culture were optimized using response surface methodology. The optimum conditions for EPS extraction were predicted to be, concentration at 87.36°C, precipitation for 35.18 h at pH 8.81, and EPS production was estimated to be 8.45 g/L. The actual yield of EPS under these conditions was 8.39 g/L. The *in vitro* antioxidant activities of the EPS were investigated and results showed that the inhibition effects of EPS at a dosage of 250 mg/L on superoxide anion radical, hydroxyl radical and reducing power were 70.63 ± 5.26, 58.22 ± 5.41 and 0.37 ± 0.02% (absorbance at 700 nm), respectively. The results provide a reference for large-scale extraction of EPS by *P. adiopsa* SX-01 in industrial fermentation.

Key words: *Pholiota adiposa* SX-01, exopolysaccharide, response surface methodology, antioxidant activity, *in vitro.*

INTRODUCTION

Pholiota adiposa [(Fr.) Quel.] is one of the artificially cultivated and precious mushrooms in many countries. It is rich in protein, essential amino acids, dietary fiber, trace elements, vitamins, and carbonhydrates (Huang et al., 2003). The polysaccharides from the fruiting bodies of *P. adiposa* had the functions of antitumour (Jiang et al., 2007), fatigue resistance (Kuniyoshi et al., 2003), antimicrobe (Dulger, 2004) and antioxidation (Ji et al., 2007).

Wasser (2002) reported that many kinds of exopolysaccharide (EPS) derived from filamentous fungi had potent anticancer activities and immunoregulatory properties. Compared with the polysaccharides from fruit bodies and mycelia, the EPS from fermentation broth with similar physiological and pharmacological functions is easily obtained. To harvest massive and effective EPS, many researchers have optimized the cultivation and production conditions for EPS in submerged cultures by *Pleurotus sajor-caju* (Confortin et al., 2008), *Fomes fomentarius* (Chen et al., 2008), *Tremella fuciformis* (Cho et al., 2006), *Pholiota squarrosa* (Wang et al., 2004), *Agrocybe cylindracea* (Kim et al., 2005), *Collybia maculate* (Lim et al., 2004), *Cordyceps jiangxiensis* (Xiao et al., 2004), *Cordyceps militaris* (Kim et al., 2003) and *Tremella mesenterica* (De Baets et al., 2002). Moreover, the extraction conditions of EPS of *Cordyceps brasiliensis* (Yang et al., 2007), *Pleurotus nebrodensis* (Jia et al., 2007) and *Morchella esculenta* (Meng et al., 2010) have been reported. However, the optimal parameters of EPS extraction by *P. adiposa* in submerged culture and its antioxidant activities *in vitro* have not been studied.

The Plackett-Burman design (PB) is applied for the

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selection of the extraction parameters that can be used to screen the most effective variables and their significant levels (Han et al., 2008). The response surface methodology (RSM) is a collection of statistical techniques that can be used to design experiments, build models, evaluate the effects of various factors and search the optimum conditions for the factors to achieve the desired responses (De et al., 2000). In this work, factors affecting the extraction of EPS of *P. adiposa* SX-01 were analyzed by PB experiments, and three significant variables (concentration temperature, precipitation time, and pH) were chosen to optimize the extraction conditions using RSM. In addition, *in vitro* antioxidant experiments were investigated.

MATERIALS AND METHODS

Chemicals

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

Microorganism and culture conditions

P. adiposa SX-01 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 sub-cultured every 3 months. Cultivation in liquid media was carried out in 250 ml Erlenmeyer flasks containing 100 ml of (g/L): potato, 200; glucose, 20; KH_2PO_4, 1.5, and $MgSO_4 \cdot 7H_2O$, 1 with natural pH. Flasks were inoculated with a 0.5 cm^2 mycelial block of *P. adiposa* SX-01 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 6 days.

Measurement and preparation of EPS

The *P. adiposa* SX-01 cultures were centrifuged at 3,000 g for 15 min the supernatant liquid was mixed with 3 volumes of 95% ethanol (v/v), stirred vigorously and kept at 4°C for 24 h. After centrifugation (3,000 g, 15 min), the precipitated EPS was dissolved in distilled water (60°C), and the EPS content was determined by the phenol–sulfuric acid method, using glucose as the standard (Chaplin and Kennedy, 1994). EPS 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 detect the antioxidant activities *in vitro*.

PB design for EPS extraction

Initial screening of the most significant fermentation parameters affecting EPS production by *P. adiposa* SX-01 was performed by PB design as reported by Plackett and Burman (1946). Seven variables including concentration multiple, concentration temperature, ethanol concentration, ethanol multiple, precipitation time, precipitation temperature and pH were studied in this experiment. In addition, 5 center points were added for the variables that could be assigned numerical values. The experimental design with the name, symbol code, and actual level of the variables is shown in Tables 1 and 2.

Response surface optimization for EPS extraction

The experimental design with name, symbol code, and actual level of the variables is shown in Tables 4 and 5. The test factors were coded according to the following equation:

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

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.

Superoxide radical scavenging assay

Superoxide anion radical scavenging activity was determined according to method of Stewar and Beewley (1980). The reaction mixture (3 ml) contained 13 mM methionine (MET), 10 mM riboflavin (RF), 75 µM nitroblue tetrazolium (NBT), 100 mM EDTA, 50 mM phosphate buffer (pH 7.8), and the EPS (5 to 250 mg/L). After illuminating the reaction mixture with a fluorescent lamp at 25°C for 30 min, the absorbance of the EPS was measured at 560 nm, using BHT as a positive control. The whole reaction was assembled in a box lined with aluminium foil. The scavenging rate was calculated using the following formula:

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

Where A_0 is the absorbance of the blank and A_1 is the absorbance of the EPS/BHT.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was measured according to the method of Winterbourn and Sutton (1984). The reaction mixture contained 1 ml of 0.15 M phosphate buffer saline (pH 7.4), 1 ml of 40 µg/ml safranin, 1 ml of 0.945 mM EDTA–Fe (II), 1 ml of 3% (v/v) H_2O_2, and 0.5 ml of the EPS (5 to 250 mg/L). After incubating at 37°C for 30 min, the absorbance of EPS was measured at 560 nm, using BHT as a positive control. The hydroxyl radical scavenging activity was expressed as:

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

Where A_0 is the absorbance of the blank and A_1 is the absorbance of EPS/BHT.

Determination of reducing power of EPS

The reducing power of EPS was evaluated according to the method of Oyaizu (1986) 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 EPS (5 to 250 mg/L). 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 1,200 g for 10 min. An aliquot of 2.5 ml supernatant

Table 1. Levels and codes of variables for Plackett–Burman design.

Variables	Symbol code	Coded levers		
		–1	0	1
Concentration multiple	A_1	1	2	3
Concentration temperature (°C)	A_2	70	80	90
Ethanol concentration (%)	A_3	75	85	95
Ethanol multiple	A_4	1	2	3
Precipitation time (h)	A_5	12	24	36
Precipitation temperature (°C)	A_6	4	6	8
pH	A_7	7	8	9

Table 2. Results of Plackett–Burman for EPS extraction by *P. adiposa* SX-01.

Runs	A_1	A_2	A_3	A_4	A_5	A_6	A_7	EPS content (g/L)
1	–1	–1	–1	–1	–1	–1	–1	6.92
2	1	–1	1	–1	1	1	–1	7.09
3	1	–1	–1	1	1	1	–1	6.73
4	–1	1	1	–1	1	1	1	7.29
5	–1	1	1	1	1	–1	–1	6.74
6	1	1	–1	–1	1	–1	1	6.89
7	–1	–1	–1	1	1	–1	1	6.68
8	–1	1	–1	–1	–1	1	–1	7.17
9	1	1	–1	1	–1	1	1	7.34
10	1	1	1	1	–1	–1	–1	6.98
11	–1	–1	1	1	–1	1	1	6.69
12	1	–1	1	–1	–1	–1	1	6.22
13	0	0	0	0	0	0	0	7.22
14	0	0	0	0	0	0	0	7.21
15	0	0	0	0	0	0	0	7.19
16	0	0	0	0	0	0	0	6.91
17	0	0	0	0	0	0	0	7.06

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 EPS was measured at 700 nm, using BHT as a positive control.

Statistical analysis

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

RESULTS AND DISCUSSION

Determination of parameters of EPS extraction

Table 2 showed that the maximum EPS yield was 7.34 g/L, while the optimal extraction parameters were: concentration multiple, 3; concentration temperature, 90°C; ethanol concentration, 75%; ethanol multiple, 3; precipitation time, 12 h; precipitation temperature, 8°C and pH 9. ANOVA results indicated that concentration temperature, precipitation time and pH had a highly significant influence on EPS extraction at the 1% level (Table 3). The influence of other parameters was not significant ($P > 0.05$). Therefore, these three important factors were chosen to optimize the process of EPS extraction using RSM.

Response surface optimization of EPS extraction

Based on the results of the PB tests, concentration temperature, precipitation time, and pH were chosen for optimization of EPS extraction by the Box–Behnken design. The experiments were planned to obtain a quadratic model consisting of 12 runs and 5 center points. The range and levels of three independent

Table 3. ANOVA for the evaluation of the regression model in Plackett–Burman design.

Parameter	Coefficients	*F*–value	*P*
Intercept	7.895	–	–
Concentration multiple	0.027	1.4275	0.2773
Concentration temperature (°C)	0.237	112.4387	< 0.0001**
Ethanol concentration (%)	0.007	0.0892	0.7752
Ethanol multiple	0.028	1.6115	0.2513
Precipitation time (h)	0.108	23.5595	0.0028**
Precipitation temperature (°C)	0.037	2.6989	0.1515
pH	0.125	31.3662	0.0014**

*Significant at 5 % level; **Significant at 1 % level.

Table 4. Levels and codes of variables for Box–Behnken design.

Variables	Symbol		Coded levels		
	Uncoded	Coded	–1	0	1
Concentration temperature (°C)	X_1	x_1	70	80	90
Precipitation time (h)	X_2	x_2	12	24	36
pH	X_3	x_3	7	8	9

Table 5. Experimental and predicted values of EPS based on Box–Behnken design.

Runs	x_1	x_2	x_3	EPS content (g/L)	
				Experimental	Predicted
1	–1	–1	0	7.89	7.91
2	1	–1	0	7.83	7.93
3	–1	1	0	8.12	8.07
4	1	1	0	8.31	8.30
5	–1	0	–1	7.87	7.84
6	1	0	–1	7.96	8.01
7	–1	0	1	8.18	8.16
8	1	0	1	8.26	8.24
9	0	–1	–1	7.79	7.75
10	0	1	–1	8.11	8.07
11	0	–1	1	8.02	8.08
12	0	1	1	8.24	8.30
13	0	0	0	8.25	8.20
14	0	0	0	8.13	8.20
15	0	0	0	8.19	8.20
16	0	0	0	8.22	8.20
17	0	0	0	8.24	8.20

variables are shown in Table 4. The Box–Behnken design matrix together with the experimental and predicted EPS data is shown in Table 5, while adequacy and fitness were evaluated by ANOVA (Table 6).

By using multiple regression analysis, the polynomial model for an empirical relationship between the extraction rate of EPS and test variables in coded units was expressed by Equation 5:

$$Y_{EPS} = 8.2 + 0.064x_1 + 0.141x_2 + 0.138x_3 - 0.05x_1x_2 - 0.018x_1x_3 - 0.025x_2x_3 - 0.065x_1^2 - 0.083x_2^2 - 0.065x_3^2 \quad (5)$$

Where Y_{EPS} is the predicted response for the yield of EPS (g/l), and x_1, x_2 and x_3 are the coded test variables for concentration temperature (°C), precipitation time (h) and pH, respectively.

The *F* value and *P* value were used to check the significance of each coefficient, which also indicated the interaction strength between independent variables. The larger the magnitude of the *F* value and smaller the *P* value, the more significant is the corresponding coefficient (Elibol, 2004). It can be seen from Table 6 that the linear term regression coefficients (x_1, x_2, x_3), the quadratic coefficients (x_1^2, x_2^2, x_3^2) and the cross product codfficient (x_1x_2) were significant at the 1% level, and the cross product coefficient (x_2x_3) was significant at the 5% level. The results indicated that the concentration temperature (°C), precipitation time (h) and pH are all significantly correlated with the yield of EPS extraction.

The ANOVA results for the effect of parameters on EPS extraction (Table 6) demonstrated that the model was highly significant ($P < 0.0001$) with a very high *F*-value (201.73). The value of correlation coefficient (R = 0.9955) indicated good agreement between the experimental and predicted values of EPS. The value of determinations coefficient (R^2) was 0.9962, indicating a good agreement between experimental and predicted values which can explain 99.62% variability of the responses. The value of adjusted determinant coefficient (adj-R^2) was 0.9912, suggesting that the total variation of 99% for EPS is attributed to the independent variables and only nearly 1% of the total variation cannot be explained by the model. The *F*-value (26.55) and *P*-value (0.0505) of lack-of-fit implied that it was not significant relative to the pure error, which indicated that the model equation was appropriate to predict the yield of EPS extraction under any combination of values.

To determine optimal levels of the test variables for the yield of EPS extraction, the 3D response surface described by the regression model is presented in Figure 1. By solving the inverse matrix (from Equation 5, the optimal values of the variables affecting the amount of EPS extraction given by the software were concentration temperature 87.36°C, precipitation time 35.18 h and pH 8.81. Under these optimal conditions, the model gave the maximum predicted values of EPS extraction (8.45 g/L), slightly higher than that obtained from the plot analysis (8.40 g/L). In view of the operating convenience, the optimal extraction parameters were determined to be

Table 6. ANOVA for the evaluation of the quadratic model.

Source	Coefficients	S.E.	Sum of squares	Mean square	*F*-value	*P*
Model	–	–	0.4300	0.0477	201.73	<0.0001**
Intercept	8.200	0.0069	–	–	–	–
x_1 (temperature)	0.064	0.0054	0.3300	0.0325	137.52	<0.0001**
x_2 (time)	0.138	0.0069	0.1500	0.1525	639.73	<0.0001**
x_3 (pH)	0.141	0.0054	0.1600	0.1596	675.10	<0.0001**
x_1x_2	0.050	0.0054	0.0100	0.0010	42.30	0.0003**
x_1x_3	–0.018	0.0054	0.0012	0.0012	5.18	0.0570
x_2x_3	–0.025	0.0077	0.0025	0.0025	10.57	0.0140*
x_1^2	–0.065	0.0077	0.0179	0.0179	75.82	<0.0001**
x_2^2	–0.0.83	0.0077	0.0288	0.0288	121.95	<0.0001**
x_3^2	–0.065	0.0077	0.0179	0.0018	75.82	<0.0001**
Lack-of-fit			0.0014	0.0005	26.55	0.0505

R^2 = 0.9962; adj-R^2 = 0.9912; R = 0.9955; *Significant at 5 % level; ** Significant at 1 % level.

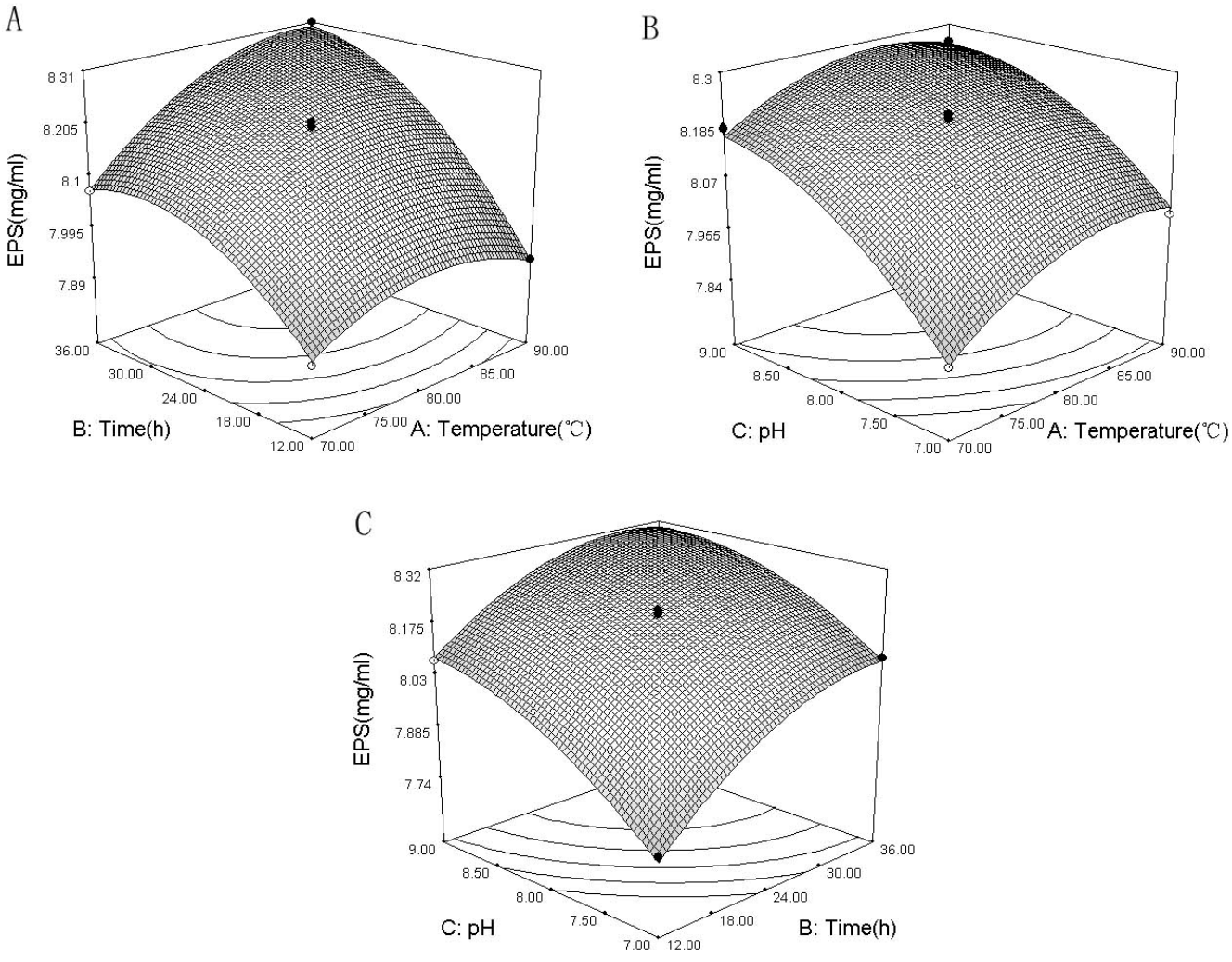

Figure 1. Response surface plot for the yield of EPS extraction by *P. adiposa* SX-01 in submerged culture in terms of the effects of (A) time and temperature, (B) pH and temperature, and (C) pH and time. Factors that were not included in the axes were fixed at their respective optimum levels.

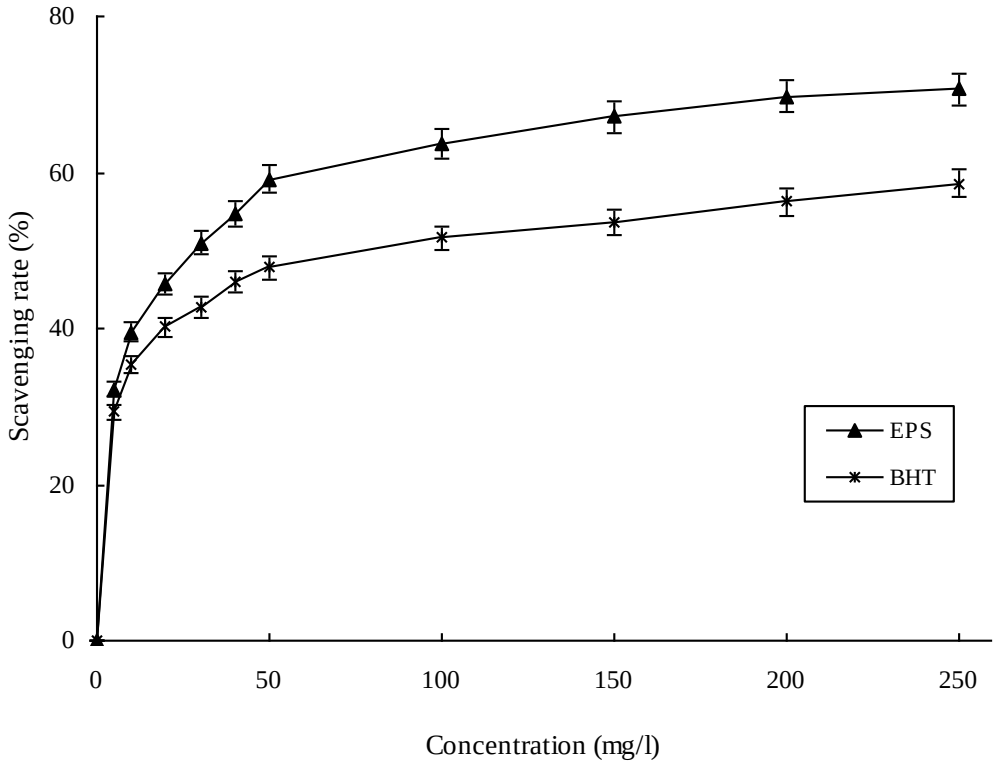

Figure 2. Scavenging effect of EPS on superoxide anion radical *in vitro*.

concentration temperature 87°C, precipitation time 35 h and pH 8.8, while the predicted value of EPS extraction was 8.43 g/L, slightly lower than that of the maximum predicted value (8.45 g/l).

Triplicate experiments were performed under the determined conditions and the value of EPS extraction (8.39 g/l) in agreement with the predicted value (8.43 g/l) was obtained, which was much higher than 2.77 g/l of *C. brasiliensis* (Yang et al., 2007), 2.40 g/l of *P. nebrodensis* (Jia et al., 2007), and 5.32 g/l of *M. esculenta* (Meng et al., 2010), respectively. The results indicated that the model was adequate for EPS extraction process.

Antioxidant activities of EPS *in vitro*

Antioxidant activities have been attributed to various reactions and mechanisms, such as radical scavenging, reductive capacity and prevention of chain initiation, binding of transition metal ion catalysts, etc. (Frankel and Meyer, 2000). In this experiment, the *in vitro* antioxidative capacities of EPS were evaluated using different biochemical methods of reducing power activity, hydroxyl radical and superoxide anion radical scavenging assay.

The superoxide anion radical scavenging activities of EPS and BHT were concentration-dependent at the dosage range of 5–250 mg/l (Figure 2). The inhibition percentage of EPS of *P. adiposa* SX-01 at 250 mg/l was 70.63 ± 5.26% ($P < 0.01$), which was 20.63 ± 1.87% higher than BHT (58.55 ± 4.31%, $P < 0.01$). It was also higher than 62.45% of *Agaricus blazei* (Zhang et al., 2004), 63.16% of *Marasmius androsaceus* (Wang et al., 2006), and 60.42% of *M. esculenta* (Meng et al., 2010), respectively. The EC_{50} value of EPS was 28.65 ± 2.55 mg/l ($P < 0.01$), 153.01 ± 14.53% lower than that of BHT (72.51 ± 6.26 mg/L, $P < 0.05$), indicating that the EPS significantly affects the scavenging of the superoxide radical.

The results of hydroxyl radical scavenging assay are described in Figure 3. The scavenging rate of EPS of *P. adiposa* SX-01 at 250 mg/L reached 58.22 ± 5.41% ($P < 0.01$), which was obviously higher than 49.36 ± 4.58% of BHT ($P < 0.05$), 23.61% of *A. blazei* (Zhang et al., 2004), and 12.44% of *Ganoderma lucidum* (Zhang and Zhang, 2005), respectively. Wang et al. (2006) and Meng et al. (2010) reported that the EC_{50} values of EPS at 250 mg/l from *M. androsaceus* and *M. esculenta* were 140.19 mg/l and 165.80 mg/l, respectively. In this study, the EC_{50} value was 119.24 ± 10.05 mg/l ($P < 0.01$), which was significantly lower than that reported earlier.

It can be seen from Figure 4 that the reducing power(absorbance at 700 nm) of EPS was 0.37 ± 0.02 ($P < 0.01$) at a dose of 250 mg/l, remarkably higher than that of BHT (0.26 ± 0.02, $P < 0.05$), showing that the EPS of *P. adiposa* SX-01 has potential antioxidant activities.

In conclusion, response surface methodology using second-order regression for a three-factor-three-level

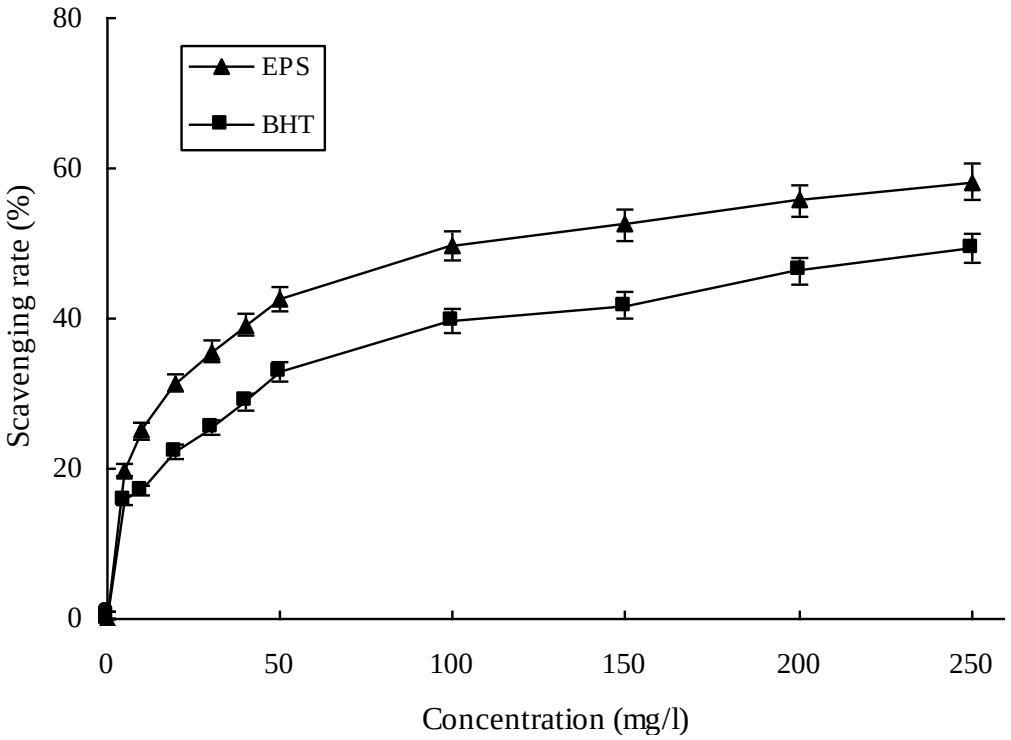

Figure 3. Scavenging effect of EPS on hydroxyl radical *in vitro*.

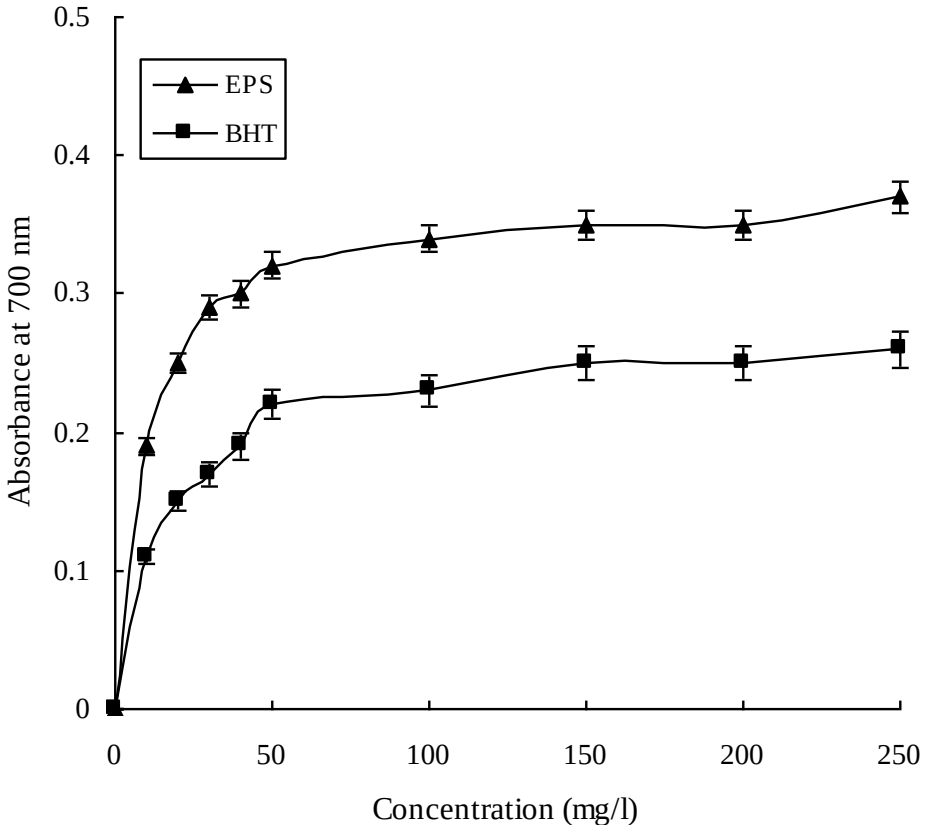

Figure 4. Reducing power of EPS.

Box-Behnken design was a successful tool for extraction optimization of EPS produced by *P. adiposa* SX-01 in submerged culture. The EPS showed antioxidative activities *in vitro*. The results provide a reference for large-scale extraction of EPS by *P. adiposa* SX-01 in industrial fermentation and the EPS can be used as a potential antioxidant which enhances adaptive immune responses.

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