

Research Paper

Extraction and *in vitro* antioxidant activity of exopolysaccharide by *Pleurotus eryngii* SI-02

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Abstract

The extraction parameters for *Pleurotus eryngii* SI-02 exopolysaccharide (EPS) produced during submerged culture were optimized using response surface methodology (RSM). The optimum conditions for EPS extraction were predicted to be, precipitation time 20.24 h, ethanol concentration 89.62% and pH 8.17, and EPS production was estimated at 7.27 g/L. The actual yield of EPS under these conditions was 7.21 g/L. The *in vitro* antioxidant results of the EPS showed that the inhibition effects of EPS at a dosage of 400 mg/L on hydroxyl, superoxide anion and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals were $59.63 \pm 3.72\%$, $38.69 \pm 2.59\%$, and $66.36 \pm 4.42\%$, respectively, which were $12.74 \pm 1.03\%$, $8.01 \pm 0.56\%$, and $12.19 \pm 1.05\%$ higher than that of butylated hydroxytoluene (BHT), respectively. The reducing power of EPS of *P. eryngii* SI-02 was 0.98 ± 0.05 , $60.66 \pm 5.14\%$ higher than that of BHT. The results provide a reference for large-scale production of EPS by *P. eryngii* SI-02 in industrial fermentation and the EPS can be used as a potential antioxidant which enhances adaptive immune responses.

Key words: *Pleurotus eryngii* SI-02, exopolysaccharide, extraction, antioxidant activity, *in vitro*.

Introduction

Pleurotus eryngii, one of the artificially cultivated and precious mushrooms in many countries, contains many biological bioactive materials, such as protein, essential amino acids, dietary fiber, trace elements, vitamins, and carbohydrates (Liu *et al.*, 2005). Polysaccharides from *P. eryngii* fruiting bodies have potential antioxidation, anti-tumor, antiviral, and immunomodulating properties (Stajic *et al.*, 2009).

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 ob-

tained. Many reports concerning to the EPS are mainly focused on the cultivation and production conditions in submerged culture by *Pleurotus sajor-caju* (Confortin *et al.*, 2008), *Tremella fuciformis* (Cho *et al.*, 2006), *Collybia maculate* (Lim *et al.*, 2004), *Cordyceps jiangxiensis* (Xiao *et al.*, 2004), and *C. militaris* (Kim *et al.*, 2003). Moreover, the extraction conditions of EPS of *P. nebrodensis* (Jia *et al.*, 2007), *Morchella esculenta* (Meng *et al.*, 2010), *Pholiota squarrosa* (Wang and Lu, 2004), *Coprinus comatus* (Hu *et al.*, 2007), *Grifola frondosa* (Cui *et al.*, 2006), *Auricularia auricula* (Zhang *et al.*, 2007) and *Ganoderma atrum* (Ye *et al.*, 2009) have been reported. However, the extraction parameters of EPS by *P. eryngii* in submerged culture and its antioxidant activities *in vitro* have not been studied.

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In this work, factors affecting the extraction of EPS of *P. eryngii* SI-02 were analyzed by Plackett-Burman (PB) experiments, and three significant variables (precipitation time, ethanol concentration and pH) were chosen to optimize the extraction conditions using response surface methodology (RSM). In addition, the *in vitro* antioxidant activities of EPS were evaluated with the hydroxyl, superoxide anion, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and reducing power as main index.

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 liquid culture

A strain of *P. eryngii* SI-02 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): potato, 200; glucose, 20; yeast extract, 3; peptone, 0.3; KH₂PO₄, 1.5, and MgSO₄·7H₂O, 1 with natural pH. Flasks were inoculated with a 0.5-cm² mycelial block of *P. eryngii* SI-02 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 8 days.

Measurement and preparation of EPS

The *P. eryngii* SI-02 cultures were centrifuged at 3000 g for 15 min, the supernatant liquid was mixed with 4 volumes of 95% ethanol (v/v), stirred vigorously and kept at 4 °C for 24 h. After centrifugation (3000 g, 15 min), the precipitated EPS was dissolved in distilled water (60 °C) for 1 h, 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 experiments for EPS extraction

Initial screening of the most significant parameters affecting EPS production by *P. eryngii* SI-02 was performed by PB design as reported by Plackett and Burman (1946). Seven variables were studied in the experiment including concentration temperature, concentration multiple, ethanol concentration, ethanol multiple, precipitation temperature, precipitation time and pH. 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

Precipitation time, ethanol concentration and pH selected by PB tests were taken into consideration for the optimization of EPS production by the Box-Behnken design. The experimental design with name, symbol code, and actual level of the variables is shown in Tables 3 and 4. The test factors were coded according to the following equation:

$$x_i = (X_i - X_0) / \Delta X_i \quad i = 1, 2, 3, \dots, k \quad (1)$$

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:

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

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.

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 110 µg/mL safranin, 2 mL of 6 mM EDTA-Fe (II), 1 mL of 3% (v/v) H₂O₂, and 0.5 mL of the EPS (50-400 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 EC₅₀ value (mg/L) of EPS or BHT is the effective concentration at which the hydroxyl radicals were scavenged by 50%. The hydroxyl radical scavenging activity was expressed as:

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

Variables	Symbol code	-1	Coded levels	1
Concentration temperature (°C)	A ₁	70	80	90
Concentration multiple	A ₂	2	3	4
pH	A ₃	5	7	9
Ethanol concentration (%)	A ₄	75	85	95
Ethanol multiple	A ₅	2	3	4
Precipitation temperature (°C)	A ₆	4	8	12
Precipitation time (h)	A ₇	12	18	24

Table 2 - Results of Plackett-Burman for EPS extraction by *Pleurotus eryngii* SI-02.

Runs	A ₁	A ₂	A ₃	A ₄	A ₅	A ₆	A ₇	EPS yield (g/L)
1	1	1	-1	1	1	1	-1	5.10
2	-1	1	1	-1	1	1	1	5.33
3	1	-1	1	1	-1	1	1	6.15
4	-1	1	-1	1	1	-1	1	4.77
5	-1	-1	1	-1	1	1	-1	3.72
6	-1	-1	-1	1	-1	1	1	5.11
7	1	-1	-1	-1	1	-1	1	3.10
8	1	1	-1	-1	-1	1	-1	4.07
9	1	1	1	-1	-1	-1	1	5.06
10	-1	1	1	1	-1	-1	-1	5.32
11	1	-1	1	1	1	-1	-1	3.56
12	-1	-1	-1	-1	-1	-1	-1	3.22
13	0	0	0	0	0	0	0	5.09
14	0	0	0	0	0	0	0	5.02
15	0	0	0	0	0	0	0	5.17
16	0	0	0	0	0	0	0	5.12
17	0	0	0	0	0	0	0	5.14
Significant			**	*			*	

* p < 0.05. ** p < 0.01.

Table 3 - Levels and codes of variables for Box-Behnken design.

Variables	Symbol		Coded	levels		
	Uncoded	Coded		-1	0	1
Precipitation time (h)	X ₁	x ₁	12	18	24	
Ethanol concentration (%)	X ₂	x ₂	75	85	95	
pH	X ₃	x ₃	5	7	9	

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

where A₀ is the absorbance of the control and A₁ is the absorbance of EPS/BHT.

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 RF, 75 μM NBT, 100 mM EDTA, 50 mM phosphate buffer (pH 7.8), and the EPS (50-400 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:

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

where A₀ is the absorbance of the blank and A₁ is the absorbance of the EPS/BHT.

DPPH scavenging assay

The DPPH scavenging activity of EPS was measured according to the method of Liu and Zhao (2006). The reaction mixture contained 2 mL of 95% ethanol, 0.1 μM DPPH and 2 mL of the EPS (50-400 mg/L). The solution was incubated at 25 °C for 15 min, and the absorbance of EPS was determined at 517 nm. The antioxidant activity of EPS was evaluated according to the following formula:

$$\text{Scavenging rate (\%)} = [(A_0 - A_1)/A_0] \times 100 \quad (5)$$

where A₁ was absorbance of EPS and A₀ was the absorbance of the DPPH solution.

Determination of reducing power

The reducing power of EPS was evaluated according to the method of Oyaizu (1986) with slightly modification. The reaction mixtures contained 2.5 mL phosphate buffer

Table 4 - Experimental and predicted values of EPS based on Box-Behnken design.

Runs	x_1	x_2	x_3	EPS yield (g/L)	
				Experimental	Predicted
1	-1	-1	0	3.63	3.45
2	1	-1	0	4.36	4.21
3	-1	1	0	5.27	5.41
4	1	1	0	6.84	7.01
5	-1	0	-1	3.66	3.87
6	1	0	-1	4.97	5.15
7	-1	0	1	5.98	5.79
8	1	0	1	7.08	6.87
9	0	-1	-1	3.03	2.98
10	0	1	-1	5.30	4.94
11	0	-1	1	4.03	4.38
12	0	1	1	7.13	7.18
13	0	0	0	6.94	6.35
14	0	0	0	6.16	6.35
15	0	0	0	5.99	6.35
16	0	0	0	6.73	6.35
17	0	0	0	5.92	6.35

(pH 6.6, 0.2 M), 2.5 mL potassium ferricyanide (1%, w/v) and the EPS (50-400 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 1200xg for 10 min. An aliquot of 2.5 mL supernatant was collected and mixed with 2.5 mL deionized water and 0.5 mL FeCl₃ (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.

Table 5 - ANOVA for the evaluation of the quadratic model.

Source	Coefficients	S.E.	Sum of squares	Mean square	F-value	p
Model	-	-	27.08	3.01	15.57	< 0.001**
Intercept	6.35	0.20	-	-	-	-
x_1 (time)	0.59	0.16	2.77	2.77	14.35	0.007**
x_2 (concentration)	1.19	0.16	11.26	11.26	58.25	< 0.001**
x_3 (pH)	0.91	0.16	6.59	6.59	34.09	< 0.006**
x_1x_2	0.21	0.22	0.18	0.18	0.91	0.371
x_1x_3	-0.05	0.22	0.01	0.01	0.06	0.818
x_2x_3	0.21	0.22	0.17	0.17	0.89	0.377
x_1^2	-0.39	0.21	0.63	0.63	3.27	0.113
x_2^2	-0.94	0.21	3.70	3.70	19.15	0.003**
x_3^2	-0.54	0.21	1.23	1.23	6.35	0.004**
Lack-of-fit			0.52	0.17	3.82	0.545

$R^2 = 0.9524$. $adj-R^2 = 0.9812$. $R = 0.9759$. * $p < 0.05$. ** $p < 0.01$.

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 value of EPS extraction was 6.15 g/L, while the optimal extraction parameters were concentration temperature 90 °C, concentration multiple 2, pH 9, ethanol concentration 95%, ethanol multiple 2, precipitation temperature 12 °C and precipitation time 24 h. Precipitation time, ethanol concentration and pH had a highly significant influence on EPS extraction at the 5% or 1% level, and the influence of other parameters was not significant ($p > 0.05$). Therefore, these three factors were chosen to optimize the process of EPS extraction using RSM.

Response surface optimization of EPS extraction

The experiments were planned to obtain a quadratic model consisting of 12 runs and 5 center points. The range and levels of three independent variables are shown in Table 3, and the Box-Behnken design matrix together with the experimental and predicted EPS data is shown in Table 4, while adequacy and fitness were evaluated by ANOVA (Table 5).

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):

$$Y_{EPS} = 6.35 + 0.59x_1 + 1.19x_2 + 0.91x_3 + 0.21x_1x_2 - 0.05x_1x_3 + 0.21x_2x_3 - 0.39x_1^2 - 0.94x_2^2 - 0.54x_3^2 \quad (6)$$

where Y_{EPS} is the predicted response for the yield of EPS (mg/L), and x_1 , x_2 and x_3 are the coded test variables for precipitation time (h), ethanol concentration (%) and pH, respectively.

It can be seen from Table 5 that the linear term regression coefficients (x_1 , x_2 , x_3) and the quadratic coefficients (x_2^2 , x_3^2) were significant at the 1% level, indicating that the precipitation time, ethanol concentration and pH are all significantly correlated with the yield of EPS extraction. The model was also significant ($p < 0.0001$) with a high F -value (15.57). The value of correlation coefficient ($R = 0.9759$) indicated good agreement between the experimental and predicted values of EPS, and R^2 (determination coefficient) was 0.9524, showing a good agreement between experimental and predicted values which can explain 95.24% variability of the responses. The value of adjusted determinant coefficient ($adj-R^2$) was 0.9812, suggesting that the total variation of 98.12% for EPS is attributed to the independent variables and only nearly 2% of the total variation cannot be explained by the model. The F -value (3.82) and p -value (0.545) 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 EPS yield, the 3D response surface described by the regression model is presented in Figure 1. The optimal values of the variables affecting the amount of EPS extraction given by the software were precipitation for 20.24 h, ethanol concentration 89.62% and pH 8.17. Under these optimal conditions, the model gave the maximum predicted values of EPS extraction (7.27 g/L). In the view of the operating convenience, the optimal extraction parameters were determined to be precipitation time 20 h, ethanol concentration 90% and pH 8.2, while the predicted value of EPS extraction was 7.18 g/L, slightly lower than that of the maximum predicted value (7.27 g/L).

Triplicate experiments were performed under the determined conditions and the value of EPS extraction (7.21 g/L) in agreement with the predicted value (7.27 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), 5.32 g/L of *M. esculenta* (Meng *et al.*, 2010), 1.05 g/L of *P. squarrosa* (Wang and Lu, 2004), 1.46 g/L of *C. comatus* (Hu *et al.*, 2007), 1.33 g/L of *G. frondosa* (Cui *et al.*, 2006), 1.67 g/L of *A. auricula* (Zhang *et al.*, 2007), and 5.23 g/L of *G. atrum* (Ye *et al.*, 2009), respectively. The results indicated that the model was adequate for EPS extraction process.

Antioxidant activity of EPS

Free radicals are known to be the major cause of various chronic and degenerative diseases, including aging, coronary heart disease, inflammation, stroke, diabetes mellitus and cancer (Cheng *et al.*, 2003; Slater, 1984). The

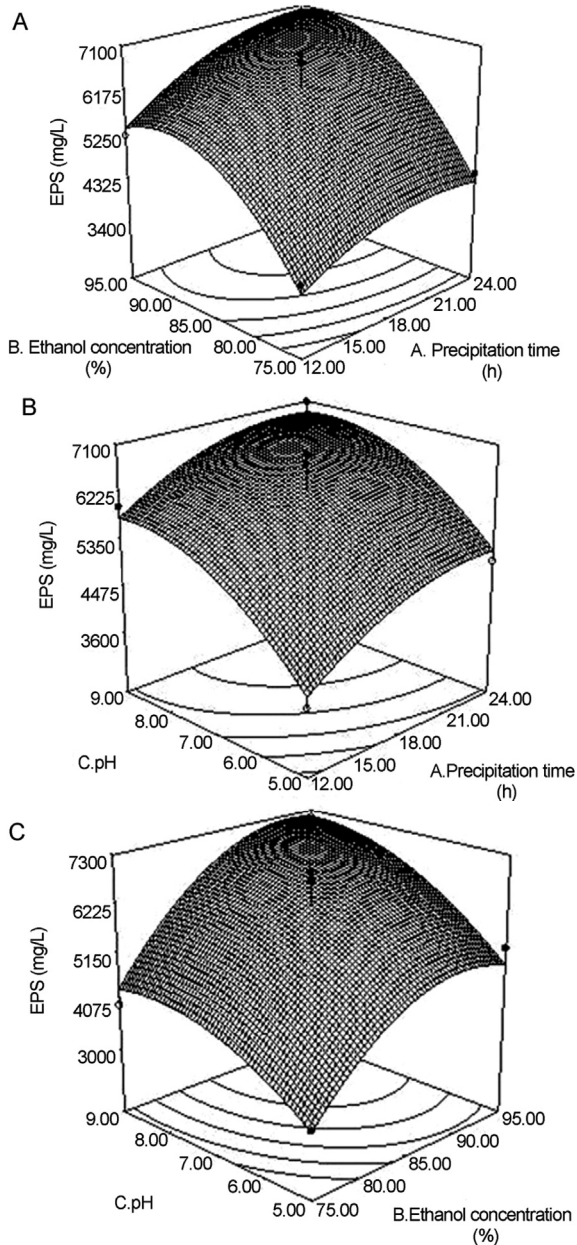


Figure 1 - Response surface plot for the yield of EPS extraction by *P. eryngii* SI-02 in submerged culture in terms of the effects of (A) ethanol concentration and precipitation time, (B) pH and precipitation time, and (C) pH and ethanol concentration. Factors that were not included in the axes were fixed at their respective optimum levels.

antioxidant compounds play an important role in preventing and curing chronic inflammation, atherosclerosis, cancer and cardiovascular disorders (Kohen and Nyska, 2002).

Hydroxyl radicals are main reactive oxygen free radicals in living organisms, which are the important reasons for causing the general processes of aging and tissue damage, and could influence the evolution of many degenerative diseases (In *et al.*, 2002). As shown in Figure 2A, the scavenging rate of EPS of *P. eryngii* SI-02 reached 59.63 ±

3.72% ($p < 0.01$) at a dosage of 400 mg/L, which was $12.74 \pm 1.03\%$ higher than that of BHT ($52.89 \pm 3.48\%$, $p < 0.01$). It was also higher than 2.3% of *G. atrum* (Ye et al., 2009), 33.4% of *Agaricus blazei* (Zhang et al., 2004), 3.5% of *Boletus edulis* (Kan et al., 2009), 51.2% of *P. nebrodensis* (Sheng et al., 2008), 22.3% of *P. adiposa* (Hu et al., 2007), 26.4% of *Termitomyces albuminosus* (Zhou and Xu, 2008), 5.2% of *Armillariella tabescens* (Ma et al., 2008), and 23.6% of *T. fuciformis* (Li et al., 2007), respectively. The EC_{50} value of EPS was 261.37 ± 20.43 mg/L ($p < 0.01$), which was not only $30.31 \pm 2.71\%$ lower than that of BHT (375.06 ± 31.15 mg/L, $p < 0.05$), but also obviously lower than 5.2 g/L of *B. edulis* (Kan et al., 2009), 1.9 g/L of *P. nebrodensis* (Sheng et al., 2008), 4.6 g/L of *P. adiposa* (Hu et al., 2007), 4.1 g/L of *T. albuminosus* (Zhou and Xu, 2008), and 8.7 g/L of *A. tabescens* (Ma et al., 2008), respec-

tively. The results indicated that the EPS of *P. eryngii* SI-02 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 free radicals and oxidizing agents (Athukorala et al., 2006). The results of superoxide anion radical scavenging assay are shown in Figure 2B and the inhibition activities of EPS and BHT were concentration-dependent at the dosage of (50-400 mg/L). The scavenging rate of EPS at 400 mg/L was $38.69 \pm 2.59\%$ ($p < 0.01$), which was $8.01 \pm 0.56\%$ higher than that of BHT ($35.82 \pm 3.01\%$, $p < 0.05$). Some researchers reported that the inhibition effects of EPS at 400 mg/L on superoxide anion radical were 6.4% of *B. edulis* (Kan et al., 2009), 9.7% of *P. adiposa* (Hu et al., 2007), 21.1% of *T. albuminosus* (Zhou

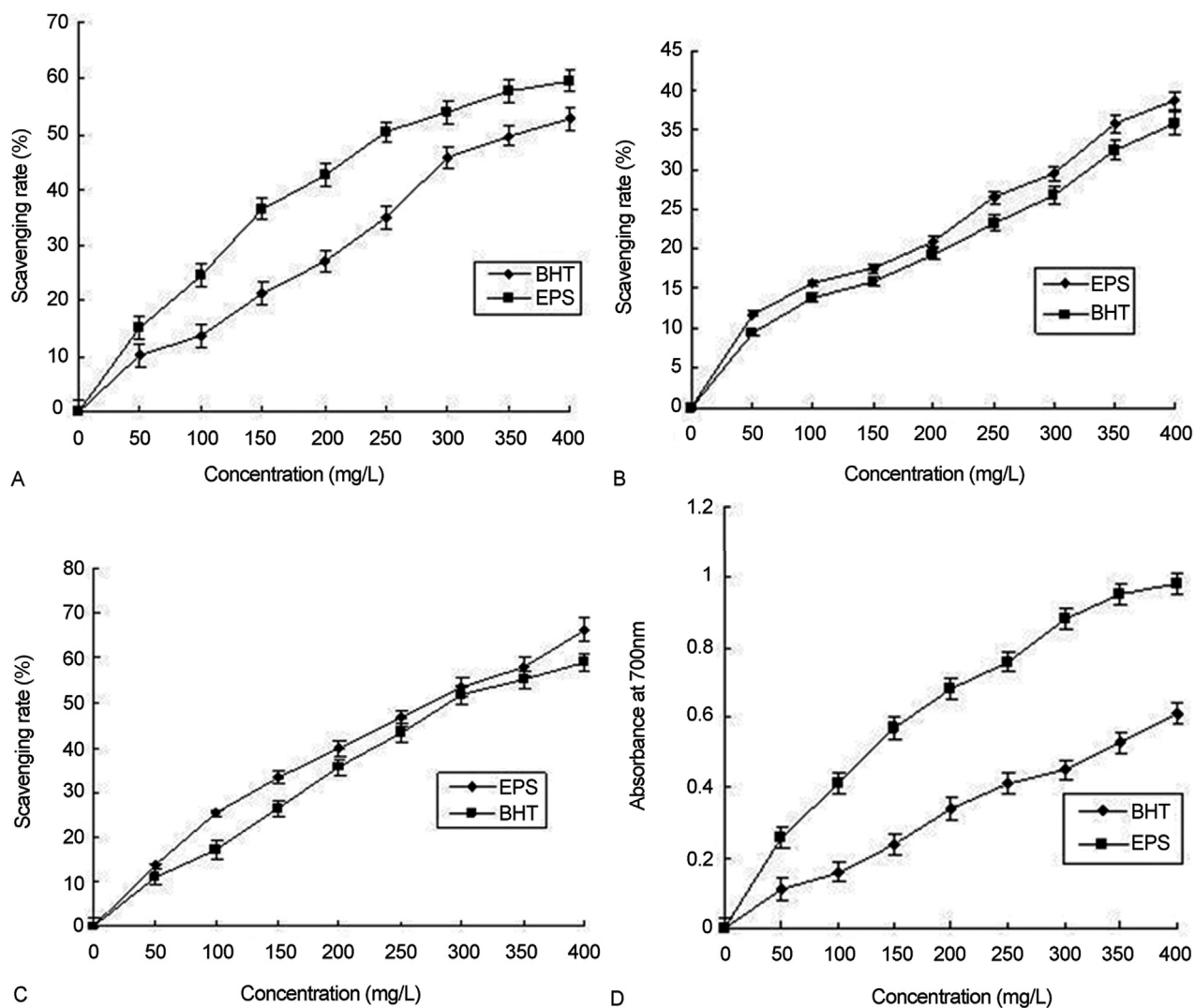


Figure 2 - Antioxidant activities of EPS of *P. eryngii* SI-02 in vitro. (A) Scavenging effect of EPS on hydroxyl radical, (B) Scavenging effect of EPS on superoxide anion radical, (C) Scavenging effect of EPS on DPPH, and (D) Reducing power of EPS.

and Xu, 2008), 6.4% of *A. tabescens* (Ma *et al.*, 2008), and 16.9% of *T. fuciformis* (Li *et al.*, 2007), respectively, markedly lower than that of EPS of *P. eryngii* SI-02 in this experiment. These data showed that the EPS of *P. eryngii* SI-02 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 (Shimada *et al.*, 1992). It can be seen from Figure 2C that the DPPH scavenging ability of EPS at 400 mg/L was $66.36 \pm 4.42\%$ ($p < 0.05$), $12.19 \pm 1.05\%$ higher than that of BHT ($59.15 \pm 3.94\%$, $p < 0.05$), which was much higher than 1.7% of *G. atrum* (Ye *et al.*, 2009), 31.6% of *Marasmius androsaceus* (Dong *et al.*, 2008), 24.5% of *T. albuminosus* (Zhou and Xu, 2008) and 4.3% of *A. tabescens* (Ma *et al.*, 2008). The EC₅₀ value of EPS was 257.21 ± 21.39 mg/L ($p < 0.01$), which was lower than that of BHT (291.06 ± 24.75 mg/L $p < 0.05$), 2.6 g/L of *T. albuminosus* (Zhou and Xu, 2008), and 2.1 g/L of *A. tabescens* (Ma *et al.*, 2008), respectively. The DPPH scavenging results revealed that the EPS probably contained substances that were proton donors and could react with free radicals to convert them to stable diamagnetic molecules.

Figure 2D showed that the reducing power (absorbance at 700 nm) of EPS at 400 mg/L was 0.98 ± 0.05 ($p < 0.01$), $60.66 \pm 5.14\%$ higher than that of BHT (0.61 ± 0.03 , $p < 0.05$). It was also much higher than 0.04 of *B. edulis* (12), 0.62 of *P. nebrodensis* (Sheng *et al.*, 2008), and 0.28 of *T. albuminosus* (Zhou and Xu, 2008), respectively. These results indicated that the EPS of *P. eryngii* SI-02 in this study has potential antioxidant capacities.

Conclusions

Response surface methodology using second-order regression for a three-factor-three-level Box-Behnken design was a successful tool for extraction optimization of EPS by *P. eryngii* SI-02 in submerged culture. The EPS showed antioxidant activities *in vitro*. The results provide a reference for large-scale extraction of EPS by *P. eryngii* SI-02 in industrial fermentation and the EPS can be used as a potential antioxidant which enhances adaptive immune responses.

Acknowledgments

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References

- Athukorala Y, Kim KN, Jeon YJ (2006) Antiproliferative and antioxidant properties of an enzymatic hydrolysate from brown alga, *Ecklonia cava*. *Food Chem Toxicol* 44:1065-1072.
- Chaplin MF, Kennedy JF (1994) *Carbohydrate Analysis, A Practical Approach*. IRL Press Ltd, USA.
- Chen DX, Liu JW, Liu ZL, Wan WH, Fang N, Xiao Y, Qi Y, Liang ZQ (2004) Optimization of submerged culture requirements for the production of mycelial growth and exopolysaccharide by *Cordyceps jiangxiensis* JXPJ 0109. *J Appl Microbiol* 96:1105-1116.
- Cheng HY, Lin TC, Yu KH, Yang CM, Lin CC (2003) Antioxidant and free radical scavenging activities of *Terminalia chebula*. *Biol Pharm Bull* 26:1331-1335.
- Cho EJ, Oh JY, Chang HY, Yun JW (2006) Production of exopolysaccharides by submerged mycelial culture of a mushroom *Tremella funiformis*. *J Biotechnol* 1:129-140.
- Confortin FG, Marchetto R, Bettin F, Camassola M, Salvador M, Dillon AJ (2008) Production of *Pleurotus sajor-caju* strain PS-2001 biomass in submerged culture. *J Ind Microbiol Biot* 35:1149-1155.
- Cui FJ, Xu HY, Xu ZH, Tao WY (2006) Optimization of the medium composition for production of mycelial biomass and exopolymer by *Grifola frondosa* GF9801 using response surface methodology. *Food and Fermentation Industries* 32:18-21.
- Dong Y, Gao X, Li TT, Zhao XH, Teng LR, Meng QF (2008) Study on *in vitro* antioxidation of *Marasmius androsaceus* extracellular polysaccharides. *Food Research and Development* 29:45-48.
- Hu M, Liang YX, Lei ZY (2007) Optimization of fermentation conditions for exopolysaccharide by *Coprinus comatus*. *Science and Technology of Food Industry* 28:157-159.
- Hu QX, Gong CY, Yan MX (2007) Antioxidation study of *Pholiota adiposa* (Fr) Quel and polysaccharide. *Journal of Central South University of Forestry and Technology* 27:58-62.
- In GK, Il LJ, Tae JO, Kug CK, Hae WS (2002) Polysaccharide-enriched fraction isolated from *Duchesnea chrysantha* protects against oxidative damage. *Biotechnol Lett* 24:1299-1305.
- Jia L, Hu SZ, Xu M (2007) Optimization of submerged culture conditions for the production of mycelial biomass and exopolysaccharide by *Pleurotus nebrodensis*. *Ann Microbiol* 57:389-393.
- Kan GS, Jiao LM, Yang YH, Chen HM (2009) Study on the antioxidative activities of extracellular polysaccharide of *Boletus edulis* Bull. *Food and Fermentation Industries* 35:57-60.
- Kim SW, Xu CP, Hwang HJ, Choi JW, Kim CW, Yun JW (2003) Production and characterization of exopolysaccharides from an entomopathogenic fungus *Cordyceps militaris* NG 3. *Biotechnol Progr* 2:428-435.
- Kohen R, Nyska A (2002) Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol Pathol* 30:620-650.
- Li YQ, Song H, Su L, Gao P, Zhang Y (2007) Research on anti-oxidative activity of polysaccharides from submerged fermentation extractum of *Tremella fuciformis*. *Journal of Fungal Research* 5:174-176.

- Liu M, Li J, Zhou B, Jia L (2005) Separation and regeneration of *Pleurotus eryngii* protoplast. *Biotechnology* 1:54-55.
- Liu X, Zhao M (2006) Antioxidant activities and functional composition content of selected *Phyllanthus emblica* fruits juice. *Food and Fermentation Industries* 5:151-154.
- Ma JB, Shen YS, Li F, Shi XQ, Zhao GH, Xie YM (2008) Study on scavenging free radical effect of polysaccharide-1b from *Armillariella tabescens*. *Edible of China* 27:38-40.
- Meng FY, Zhou B, Lin RS, Jia L, Liu XN, Deng P, Fan KM, Wang GY, Wang L, Zhang JJ (2010) Extraction optimization and *in vivo* antioxidant activities of exopolysaccharide by *Morchella esculenta* SO-01. *Bioresour Technol* 101:4564-4569.
- Oyaizu M (1986) Studies on products of browning reaction: antioxidative activities of products of browning reaction prepared from glucosamine. *Jpn J Nutri* 44:307-315.
- Plackett RL, Burman JP (1946) The design of optimum multi-factorial experiments. *Biometrika* 33:305-325.
- Sheng W, Wu P, Guo H, Liu RX (2008) Studies on anti-oxidation activity of polysaccharide and exopolysaccharide extracted from *Pleurotus nebrodensis*. *Forest By-Product and Speciality in China* 94:8-10.
- Shimada K, Fujikawa K, Yahara K, Nakamura T (1992) Antioxidative properties of xanthan on the antioxidation of soybean oil in cyclodextrin emulsion. *J Agr Food Chem* 40:945-952.
- Slater TF (1984) Free-radical mechanisms in tissue injury. *Biochem J* 222:1-15.
- Stajic M, Vukojevic J, Duletic-Lausevic, S. (2009). Biology of *Pleurotus eryngii* and role in biotechnological processes. *Crit Rev Biotechnol*. 29:55-66.
- Stewart RC, Beewley JD (1980) Lipid peroxidation associated with accelerated aging of soybean areas. *Plant Physiol* 65:245-248.
- Wang YX, Lu ZX (2004) Optimization of cultivation conditions for extracellular polysaccharide and mycelium biomass by *Pholiota squarrosa*. *Microbiology* 31:42-47.
- Wasser SP (2002) Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Appl Microbiol Biot* 60:258-274.
- Winterbourn, CC, Sutton HC (1984) Hydroxyl radical production from hydrogen peroxide and enzymatically generated paraquat radicals: Catalytic requirements and oxygen dependence. *Arch Biochem Biophys* 235:116-12
- Yang RL, Chen WD, Wu XY, Liu XM (2007) Extraction technology optimization for extra-cellular polysaccharide (EPS) of *Cordyceps brasiliensis*. *Food Science* 28:91-96.
- Ye M, Chen JS, Yang L, Tan W, Sun HJ (2009) Optimization of extraction technology of extracellular polysaccharide from fermentation broth *Ganoderma atrum* and study on its antioxidant activity *in vitro*. *Food Science* 30:47-50.
- Yun JW (2004) Optimization of medium by orthogonal matrix method for submerged mycelial culture and exopolysaccharide production in *Collybia maculate*. *Appl Biochem Biotech* 2:159-170.
- Zhang DW, Zhao L, Wu TX (2007) Optimization of *Auricularia Auricula* exopolysaccharide fermentation medium by orthogonal experiment design. *Journal of Guizhou University of Technology (Natural Science Edition)* 36:40-43.
- Zhang H, Li CB, Liu CJ (2004) Studies on anti-oxidation activity of exopolysaccharide AbEXP-Ia extracted from *Agaricus blazei* Murrill *in vitro*. *Edible Fungi of China* 24:48-49.
- Zhou JP, Xu HY (2008) Antioxidant activity of fractions from *Termitomyces albuminosus* in submerged culture. *Chinese Wild Plant Resources* 27:46-49.