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Full Length Research Paper

Extraction optimization of exopolysaccharide produced by *Pleurotus cornucopiae* SS-02 and its antioxidant activity

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The extraction parameters for *Pleurotus cornucopiae* SS-02 exopolysaccharide (EPS) produced during submerged culture were optimized using Plackett–Burman (PB) design and central composite design (CCD). The optimum conditions for EPS extraction were ethanol concentration of 86.97%, pH 8.99 and concentrated multiple 2.43, and the EPS production was estimated at 3.96 g/l. The actual yield of EPS under these conditions was 3.75 g/l. The *in vitro* antioxidant activities of the EPS showed that the inhibition effects of EPS at a dosage of 4 g/l on superoxide anion and hydroxyl radicals were 70.53 ± 3.27% and 40.59 ± 2.81%, respectively and the reducing power was 0.42 ± 0.02 (absorbance at 700 nm). The activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) in mice blood were 260.62 ± 22.18 U/ml, 187.66 ± 16.28 U/ml, and 403.75 ± 36.16 U/ml, respectively and the malondialdehyde (MDA) content was 5.01 ± 0.36 nmol/ml. The results provide a reference for large-scale extraction of EPS by *P. cornucopiae* SS-02 in industrial fermentation, and the EPS can be used as a potential antioxidant which enhances adaptive immune responses.

Key words: Exopolysaccharide, *Pleurotus cornucopiae* SS-02, antioxidant activity, *in vitro*, *in vivo*

INTRODUCTION

Pleurotus cornucopiae (Paul. ex Pers.), a nutritional and medicinal mushroom with refreshing fragrance, contains many biological active materials, such as protein, trace elements, dietary fiber, vitamins, and carbohydrates (Pavel, 2009). The polysaccharide from *P. cornucopiae* fruiting body had the functions of antitumour, immuno-regulation, fatigue resistance and antivirus (Huang 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 obtained. Many reports concerning the EPS are mainly focused on the cultivation and production conditions in submerged culture by *Cordyceps*

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Abbreviations: EPS, Exopolysaccharide; **CCD,** central composite design; **SOD,** superoxide dismutase; **GSH-Px,** glutathione peroxidase; **CAT,** Catalase; **MDA,** malondialdehyde; **PB,** Plackett–Burman.

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

Variable	Symbol code	Coded level		
		-1	0	1
Extraction time (h)	A_1	1	2	3
Extraction temperature (°C)	A_2	60	70	80
Ethanol concentration (%)	A_3	90	85	95
Ethanol multiple	A_4	3	4	5
Precipitation temperature (°C)	A_5	-4	0	4
pH	A_6	5	7	9
Precipitation time (h)	A_7	48	36	48
Concentrated multiple	A_8	1	2	3
Concentrated temperature (°C)	A_9	60	70	80

brasiliensis (Yang et al., 2007), *Lentinula edodes* (Lu et al., 2010), *Pholiota squarrosa* (Zhao et al., 2007), *Morchella esculenta* (Xu et al., 2008), *P. sajor-caju* (Confortin et al., 2008), *Tremella fuciformis* (Cho et al., 2006), *Agrocybe cylindracea* (Kim et al., 2005), *Collybia maculate* (Lim et al., 2004), *Fomes fomentarius* (Chen et al., 2008), *P. nebrodensis* (Jia et al., 2007), *Coprinus comatus* (Hu et al., 2007), *Grifola frondosa* (Cui et al., 2006), *Auricularia auricular* (Zhang et al., 2007). Moreover, the extraction conditions of EPS of *C. brasiliensis* (Yang et al., 2007), *P. nebrodensis* (Jia et al., 2007) and *M. esculenta* (Meng et al., 2010) have been reported. However, the optimal parameters of EPS extraction by *P. cornucopiae* in submerged culture and its antioxidant activities have not been studied.

The aims of this study were to optimize the extraction conditions of EPS by *P. cornucopiae* SS-02 in submerged culture on the basis of Plackett–Burman (PB) design and central composite design (CCD), and to determine the antioxidant activities of EPS *in vitro* and *in vivo*.

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

A strain of *Pleurotus cornucopiae* SS-02 was provided by our laboratory and used in this experiment. It was incubated on synthetic potato dextrose agar (PDA) plates for 7 days at 25°C, maintained at 4°C and subcultured every 2 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. cornucopiae* SS-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 7 days.

Measurement and preparation of EPS

The *P. cornucopiae* SS-02 culture was centrifuged at 3,600 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 (3,600 g, 15 min), the precipitated EPS was dissolved in deionized 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 experiments for EPS extraction

Initial screening of the most significant fermentation parameters affecting EPS production by *P. cornucopiae* SS-02 was performed by PB design (Plackett and Burman, 1946). Nine variables including extraction time, extraction temperature, ethanol concentration, ethanol multiple, precipitation temperature, pH, precipitation time, concentrated multiple and concentrated temperature were studied in this experiment. In addition, three center points were added for the variables that could be assigned numerical values. The experimental design of PB test is shown in Table 1.

CCD optimization for EPS extraction

Ethanol concentration, pH and concentration multiple selected by PB tests were taken into consideration for the optimization of EPS production by CCD. The experimental design with name and symbol code is shown in Table 2. The test factors were coded according to the following equation:

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

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.

Table 2. Levels and codes of variables for central composite design.

Variable	Symbol		Coded level		
	Uncoded	Coded	−1	0	1
Ethanol concentration (%)	X_1	x_1	75	85	95
pH	X_2	x_2	5	7	9
Concentrated multiple	X_3	x_3	1	2	3

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 ter; β_i is the linear term; β_{ii} is the squared term; β_{ij} is the interaction term and x_i and x_j are the coded level of independent variables.

Antioxidant activities of EPS *in vitro*

The superoxide anion scavenging activity of EPS was determined according to the method of Gülçin et al. (2011) with slight modification. The reaction mixture contained 0.5 ml of phosphate buffer saline (pH 7.8, 0.2 M), 0.3 ml of riboflavin (10 mM), 0.25 ml of methionine (13 mM), 0.25 ml of NBT (75 μM), and 1 ml of the EPS (0.4 to 4 g/l). After illuminating the reaction mixture with a fluorescent lamp at 25°C for 20 min, and the absorbance of the EPS was measured at 560 nm, using BHT as a positive control. The inhibition percentage of superoxide anion generation was calculated using the following formula:

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

Where, A_0 is the absorbance of the blank and A_1 is the absorbance of the EPS or BHT (Koksal et al. 2011).

The hydroxyl radical scavenging assay was measured according to the method of Wei et al. (2009) with slight modification. The reaction mixture contained 1 ml of phenanthroline (7.5 mM), 1 ml of $FeSO_4$ (0.75 mM), 5 ml of phosphate buffer (pH 7.4), 1 ml of 3% (v/v) H_2O_2 and 1 ml of the EPS (0.4 to 4 g/l). After incubating at 37°C for 60 min, the absorbance of EPS was measured at 560 nm, using BHT as a positive control. The hydroxyl radical scavenging activity was expressed as:

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

Where, A_0 is the absorbance of the blank and A_1 is the absorbance of EPS or BHT (Koksal et al. 2011).

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 EPS (0.4 to 4 g/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 was collected and mixed with 2.5 ml deionized water and 0.5 ml $FeCl_3$ (0.1%, w/v). After incubating at 25°C for 15 min, the absorbance of the EPS was measured at 700 nm, using BHT as a positive comparison.

Animal experiments

Forty male mice (KunMing strain), weighing 20 ± 2 g were obtained from Taibang Biological Products Co. Ltd (Taian, China) and housed in stainless steel cages under controlled conditions (temperature 22 ± 1°C, humidity 60 to 65%, lights on 12 h every day) with free access to standard food. After a 3 day acclimatization period, all animals were randomly divided into eight groups (5 in each group). The seven experiment groups received doses of 25, 50, 100, 150, 200, 300, 400 mg EPS/kg body weight of mice by filling the stomach (0.01 ml/g body weight) with a syringe. The control group received the same volume of saline. The mice were allowed to have free access to water and food for 30 day. The experiments were performed under guidance of the Shandong Agricultural University Committee. At the end of the experiment, all animals were sacrificed under ether anesthesia, and blood samples were taken from the retrobulbar vein with a vacutainer and anticoagulated by heparin (stored at –80°C). The livers, hearts, spleens, and kidneys were rapidly removed, weighed, and homogenized (1:9, w/v) immediately in 0.2 M phosphate buffer (4°C, pH 7.4), respectively. The homogenates were centrifuged (6,000*g*) at 4°C for 20 min and the supernatants were stored at –20°C.

Measurement of biochemical parameters *in vivo*

The superoxide dismutase (SOD) activities of liver, heart, spleen, kidney, and blood were measured according to the method of Bayer and Fridovich (1987) with slight modification (Gülçin et al., 2009). Fifty microliters of the organ extracts was mixed with 1 ml of physiological saline, centrifuged for 3 min at 2,500 g, and the pellet was used for the enzyme reaction. Ice-cold water (0.2 ml), 0.2 ml 95% ethanol, 0.1 ml trichloromethane were added to the pellet and mixed thoroughly with a homogenizer (Bagmixer 400, France). The mixture presented three layers after centrifugation (3,500 g, 5 min): SOD extract (top layer), haemoglobin precipitation (middle layer) and trichloromethane (bottom layer). The top layer was removed by micropipettor carefully and the mixture, including 0.05 ml of SOD extract, 50 mM potassium phosphate buffer (pH 7.0), 13 mM methionine, 0.075 mM nitroblue tetrazolium (NBT), 0.01 mM EDTA and 0.002 mM riboflavin, was reacted for 20 min. The enzyme activity was expressed in relative units per milligram protein or micromoles per minute per liter of blood. The unit of SOD activity was expressed as U (50% inhibition of photochemical reduction of NBT as 1 U).

The activity of glutathione peroxidase (GSH-Px) was measured as described by Flohé and Günzler (1984). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM EDTA, 1 mM NaN_3, 0.15 mM NADPH, 1 mM GSH, and 2.4 U/ml of glutathione reductase (GR). The reaction was initiated by adding 0.15 mM H_2O_2. The rate of NADPH consumption was recorded at 340 nm. The activity of GSH-Px was expressed as mM of NADPH oxidized per minute per milligram of tissue or micromoles per minute per milliliter of blood.

The activity of catalase (CAT) was determined by the method of Schlorff (1999) with slightly modification. The mixture, including 2 ml sodium-potassium phosphate, 65 μM of H_2O_2, and 0.5 ml of blood or

Table 3. Results of Plackett–Burman for EPS extraction by *P. cornucopiae* SS-02.

Run	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	EPS yield (g/l)
1	1	1	–1	1	1	1	–1	–1	–1	0.86
2	-1	1	1	–1	1	1	1	–1	–1	1.31
3	1	–1	1	1	–1	1	1	1	–1	2.62
4	-1	1	–1	1	1	–1	1	1	1	1.52
5	-1	–1	1	–1	1	1	–1	1	1	2.33
6	-1	–1	–1	1	–1	1	1	–1	1	1.57
7	1	–1	–1	–1	1	–1	1	1	–1	1.29
8	1	1	–1	–1	–1	1	–1	1	1	1.65
9	1	1	1	–1	–1	–1	1	–1	1	1.63
10	-1	1	1	1	–1	–1	1	1	–1	1.71
11	1	–1	1	1	1	–1	–1	–1	1	1.12
12	-1	–1	–1	–1	–1	–1	–1	–1	–1	0.70
13	0	0	0	0	0	0	0	0	0	1.18
14	0	0	0	0	0	0	0	0	0	1.30
15	0	0	0	0	0	0	0	0	0	1.16
Significant			*			**			*	

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

tissue extract was reacted at 37°C for 1 min, and then 2 ml of ammonium molybdate (32.4 mM) was added for termination reaction at 25°C. The activity of CAT was expressed as μM of H_2O_2 degraded per minute per milligram of tissue or per minute per milliliter of blood.

The content of malondialdehyde (MDA) was measured by the method of Zhao et al. (2002) with slight modification. The mixture, including 0.2 ml sample and 2 ml of 0.6% thiobarbituric acid (TBA, w/v), was heated in a boiling water for 15 min. After cooling rapidly, the mixture was centrifuged at 3,000 g for 10 min, and the supernatant was used for the determination of MDA 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. Inc., Minneapolis, USA) including ANOVA.

RESULTS AND DISCUSSION

Determination of parameters of EPS extraction

Table 3 shows that the maximum EPS yield was 2.62 g/l, while the optimal extraction parameters were extraction time 3 h, extraction temperature 60°C, ethanol concentration 95%, ethanol multiple 5, precipitation temperature –4°C, pH 9, precipitation time 48 h, concentrated multiple 3, concentrated temperature 60°C. ANOVA results indicated that ethanol concentration, pH and concentrated multiple had a highly significant influence on EPS extraction at the 5 or 1% level, and the influence of other parameters was not significant ($P > 0.05$) (Table 3). Therefore, these three important factors were chosen to optimize the process of EPS extraction using CCD.

CCD 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. The CCD 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 EPS and test variables in coded units was expressed by Equation (5).

$$Y_{EPS} = 3.5 + 0.12x_1 + 0.29x_2 + 0.12x_3 + 0.03x_1x_2 + 0.09x_1x_3 + 0.03x_2x_3 - 0.36x_1^2 - 0.1x_2^2 - 0.19x_3^2 \quad (5)$$

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 ethanol concentration (%), pH and concentrated multiple, 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. It can be seen from Table 5 that the linear term regression coefficients (x_1, x_2, x_3) and the quadratic coefficients (x_1^2, x_3^2) were significant at the 1% level, indicating that the

Table 4. Experimental and predicted values of EPS based on central composite design.

Run	x_1	x_2	x_3	EPS yield (g/l)	
				Experimental	Predicted
1	−1	−1	0	2.73	2.66
2	1	−1	0	2.82	2.85
3	−1	1	0	3.21	3.19
4	1	1	0	3.41	3.48
5	−1	0	−1	2.78	2.80
6	1	0	−1	2.91	2.86
7	−1	0	1	2.81	2.86
8	1	0	1	3.31	3.28
9	0	−1	−1	2.80	2.83
10	0	1	−1	3.39	3.36
11	0	−1	1	3.00	3.02
12	0	1	1	3.69	3.65
13	0	0	0	3.49	3.50
14	0	0	0	3.44	3.50
15	0	0	0	3.50	3.50
16	0	0	0	3.59	3.50
17	0	0	0	3.49	3.50

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

Source	Coefficient	S.E.	Sum of square	Mean square	*F*-value	*P*
Model	−	−	1.75	0.19	41.84	<0.0001**
Intercept	3.50	0.030	−	−	−	−
x_1(concentration)	0.12	0.024	0.110	0.110	22.82	0.0020**
x_2(pH)	0.29	0.024	0.690	0.690	148.89	<0.0001**
x_3(multiple)	0.12	0.024	0.110	0.110	23.32	0.0019**
x_1x_2	0.03	0.034	0.002	0.002	0.65	0.4458
x_1x_3	0.09	0.034	0.034	0.034	7.38	0.0299*
x_2x_3	0.03	0.034	0.003	0.003	0.54	0.4866
x_1^2	−0.36	0.033	0.560	0.560	119.99	<0.0001**
x_2^2	−0.10	0.033	0.039	0.039	8.37	0.0232*
x_3^2	−0.19	0.033	0.150	0.150	31.42	0.0008*
Lack-of-fit			0.021	0.007	2.31	0.2181

R=0.9908, R^2=0.9817, adj-R^2=0.9583, * Significant at 5% level. ** Significant at 1% level.

ethanol concentration (%), pH and concentration multiple are all significantly correlated with the yield of EPS extraction.

The ANOVA results for the effect of parameters on EPS extraction (Table 5) demonstrated that the model was highly significant (P < 0.0001) with a very high F-value (41.84). The value of correlation coefficient (R = 0.9908) indicated good agreement between the experimental and predicted values of EPS, and R^2 (determination coefficient) was 0.9817, showing a good agreement between experimental and predicted values which can explain 98.17% variability of the responses. The value of adjusted determinant coefficient (adj-R^2) was 0.9583, suggesting that the total variation of 95.83% for EPS is attributed to the independent variables and only nearly 5% of the total variation cannot be explained by the model. The F-value (2.31) and P-value (0.2181) 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

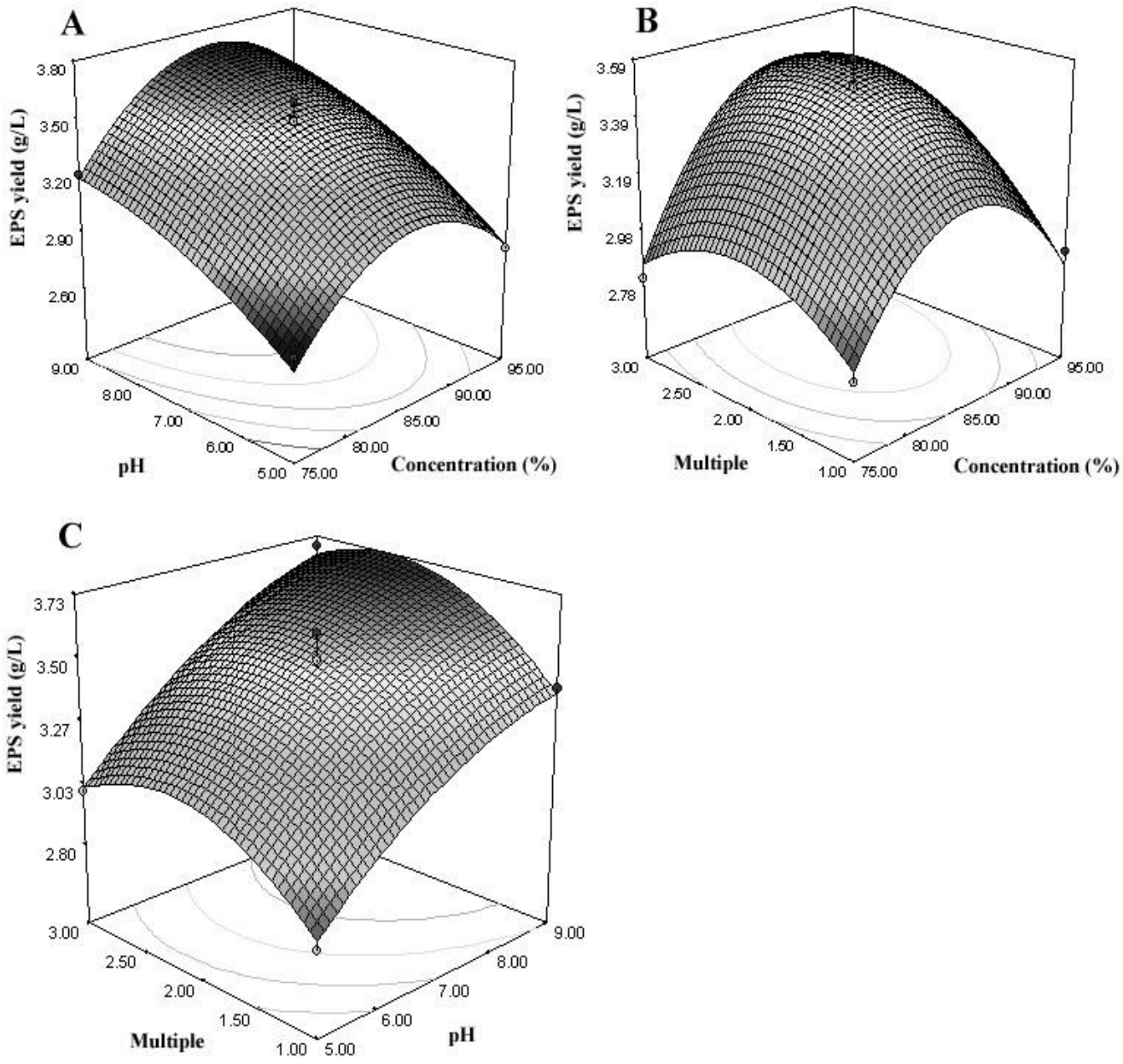

Figure 1. Response surface plot for the extraction yield of EPS of *P. cornucopiae* SS-02 in terms of the effects of (A) pH and ethanol concentration, (B) concentrated multiple and ethanol concentration, and (C) concentrated multiple and pH. Factors that were not included in the axes were fixed at their respective optimum levels.

of the variables affecting the amount of EPS extraction given by the software were ethanol concentration 86.97%, pH 8.99 and concentrated multiple 2.43. Under these optimal conditions, the model gave the maximum predicted values of EPS extraction (3.96 g/l). In the view of the operating convenience, the optimal extraction parameters were determined to be ethanol concentration 85%, pH 9 and concentrated multiple 2, while the predicted value of EPS extraction was 3.68 g/l, slightly lower than that of the maximum predicted value (3.96 g/l).

Triplicate experiments were performed under the determined conditions and the value of EPS extraction (3.75 g/l) in agreement with the predicted value (3.96 g/l) was obtained. The EPS yield was much higher than 2.78 g/l of *C. brasiliensis* (Yang et al. 2007), 2.33 g/l of L. edodes (Lu et al., 2010), 2.4 g/l of *M. esculenta* (Xu et al., 2008), 0.85 g/l of *P. squarrosa* (Zhao et al., 2007), 2.4 g/l of *P. nebrodensis* (Jia et al., 2007), 1.46 g/l of *C. comatus* (Hu et al., 2007), 1.33 g/l of *G. frondosa* (Cui et al., 2006) and 1.67 g/l of *A. auricular* (Zhang et al., 2007),

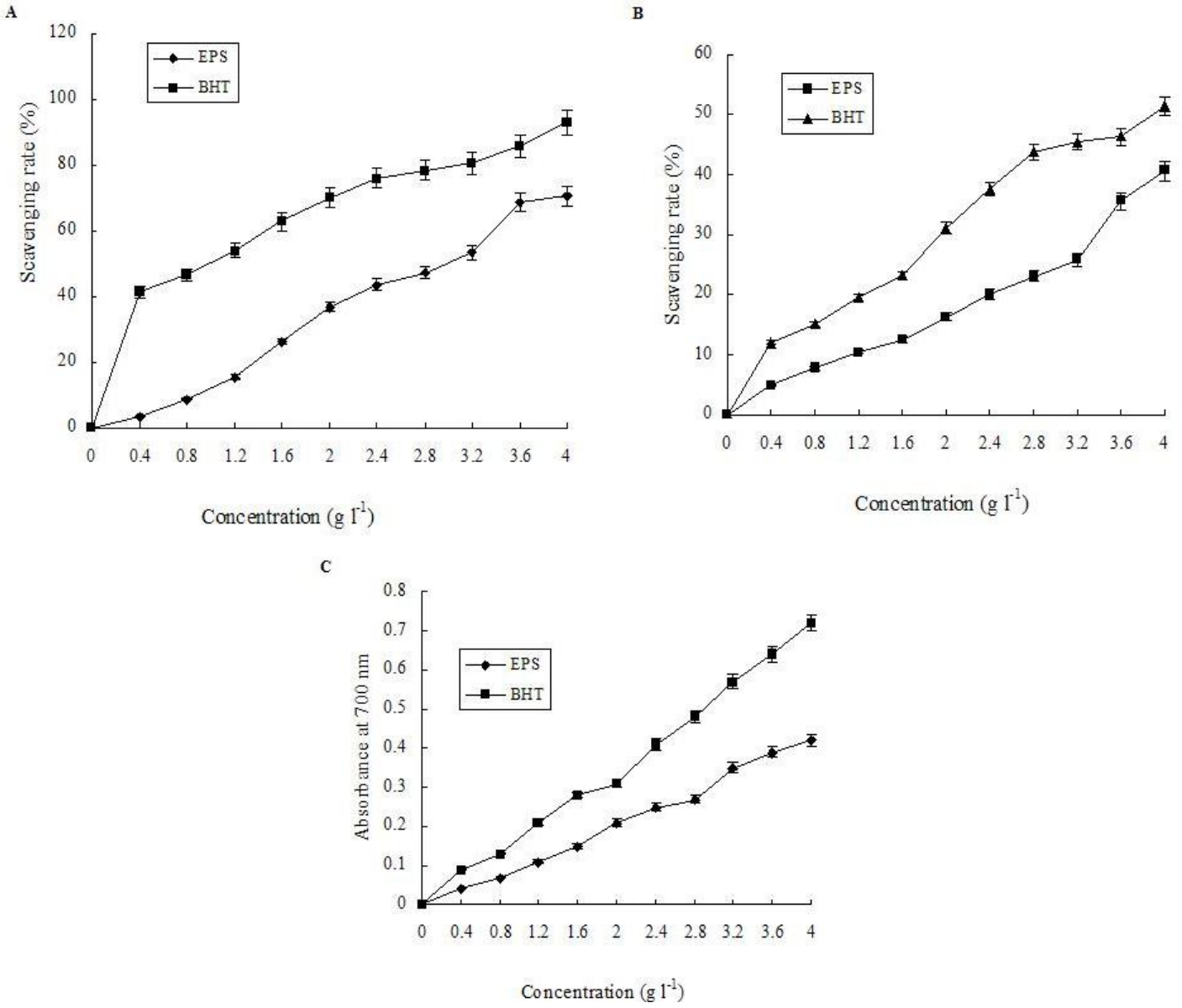

Figure 2. Antioxidant activities of EPS *in vitro*. (A) Scavenging effect of EPS on superoxide anion radical, (B) Scavenging effect of EPS on hydroxyl radical, and (C) Reducing power of EPS.

respectively. The results indicate that the model was adequate for EPS extraction process.

Antioxidant activity of EPS in vitro

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; Gülçin et al., 2010). The antioxidant compounds play an important role in preventing and curing chronic inflammation, atherosclerosis, cancer and cardiovascular disorders (Kohen and Nyska, 2002).

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 2A and the inhibition activities of EPS and BHT were concentration-dependent at the dosage of (0.4 to 4 g/l). The scavenging rate of EPS at 4 g/l was 70.53 ± 3.27% ($P < 0.01$), which

was much higher than 48.7% of *C. militaris* (Chen et al., 2009), 31.4% of *Boletus edudis* (Kan et al., 2009), 36.2% of *P. adiposa* (Hu et al., 2007), and 27.5% of *Antrodia camphorate* (Zhang et al., 2007), respectively. The EC50 value of EPS was 2.91 ± 0.15 g/l ($P < 0.01$), which was lower than 6.5 g/l of *B. edudis* (Kan et al., 2009) and 3.9 g/l of *P. adiposa* (Hu et al., 2007). The data show that the EPS of *P. cornucopiae* SS-02 can effectively protect cell from damage and lipid peroxidation.

Hydroxyl radicals are main the 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 2B, the scavenging rate of EPS of *P. cornucopiae* SS-02 reached 40.59 ± 2.81% ($p < 0.01$) at a dosage of 4 g/l, which was lower than that of BHT (51.4 ± 3.92%, $P < 0.05$). Some researchers reported that the inhibition effects of EPS at 4 g/l on hydroxyl radical were 36.5% of *C. militaris* (Chen et al., 2009), 4.7% of *Ganoderma atrum* (Ye et al., 2009), 32.8% of *B. edudis* (Kan et al., 2009), 32.4% of *P. adiposa* (Hu et al., 2007), 22.1% of *A. camphorate* (Zhang et al., 2007), and 31.7% of *G. frondosa* (Li et al., 2003), respectively markedly lower than that of EPS of *P. cornucopiae* SS-02 in this experiment. The results indicate that the EPS of *P. cornucopiae* SS-02 significantly affects the scavenging of hydroxyl radical.

Figure 2C shows that the reducing power (absorbance at 700 nm) of EPS at 4 g/l was 0.42±0.02 ($P < 0.01$), which was much higher than 0.12 of B. edudis (Kan et al., 2009), 0.31 of *T. aurantialba* (Huang et al., 2009), and 0.26 of *C. comatus* (Wu et al., 2007), respectively.

Antioxidant activities of EPS *in vivo*

SOD can eliminate the superoxide anion radical to protect cells from damage. Experiments in animals demonstrate a correlation between SOD and tolerance to oxygen toxicity (Zhang et al., 2009). It can be seen from Figure 3A that the SOD activities of 400 mg/kg group in mice heart, liver, spleen, kidney and blood reached 254.12 ± 19.26 U/mg, 246.63 ± 21.05 U/mg, 233.47±20.14 U/mg, 199.65 ± 17.58 U/mg, and 260.62 ± 22.18 U/ml, respectively which were 14.09 ± 1.17, 17.19 ± 1.26, 12.98 ± 1.02, 7.61 ± 0.53 and 30.39 ± 2.23% higher than that of the control group, respectively. The ANOVA showed that the activity of SOD in 400 mg/kg group was significant compared to that of the control group ($P < 0.01$). The EPS of *P. cornucopiae* SS-02 can effectively scavenge the superoxide anion radical by increasing SOD activity in mice blood and tissues.

GSH-Px catalyzes the reduction of hydrogen peroxide derived from oxidative metabolism as well as peroxides from oxidation of lipids and is considered the most effective enzyme against lipid peroxidation (Zhang et al., 2009). The GSH-Px activities of 400 mg/kg group in mice heart, liver, spleen, kidney and blood were 269.32 ± 23.05 U/mg, 159.45 ± 12.96 U/mg, 97.71 ± 8.15 U/mg, 213.74 ± 19.23 U/mg, and 187.66 ± 16.28 U/ml, respectively which were 18.57 ± 1.62, 26.35 ± 2.17, 28.66 ± 2.25, 16.61 ± 1.47 and 46.64 ± 3.29%, respectively (Figure 3B). The maximum GSH-Px activity appeared in liver tissue which may be related to that and the liver is the major organ to remove lipid hydroperoxide. The ANOVA showed that GSH-Px activities of heart, liver, spleen, kidney and blood of mice treated with EPS were significantly higher ($P < 0.01$). These data indicated that the EPS tested has a noticeable effect on improving GSH-Px activity.CAT is the most important enzyme to provide a homeostasis for hydrogen peroxide. Chance et al. (1979) reported that the physiological variation of CAT concentration in different organs and tissues leads to different steady-state levels of hydrogen peroxide concentration for the same rate of hydrogen peroxide generation. As shown in Figure 3C, the CAT activities of 400 mg/kg group in mice heart, liver, spleen, kidney and blood reached 380.18 ± 31.26 U/mg ($P < 0.01$), 490.77 ± 37.31 U/mg ($P < 0.05$), 210.91 ± 18.94 U/mg ($P < 0.05$), 423.76 ± 33.09 U/mg ($P < 0.01$), and 403.75 ± 36.16 U/mL ($P < 0.01$), respectively.

MDA is considered as a secondary indicator formed by lipid peroxidation, reflecting the content of free radicals produced by lipid peroxidation (Niki et al., 2005; Husain et al., 2003). Figure 3D shows that the MDA contents at 400 mg/kg group in heart, liver, spleen, kidney and blood of mice were 3.29 ± 0.18 nmol/mg, 2.06 ± 0.15 nmol/mg, 10.99±0.83 nmol/mg, 9.79±0.71 nmol/mg, and 5.01 ± 0.36 nmol/ml, respectively which were 26.90 ± 1.72, 24.91 ± 2.07, 53.19 ± 3.29%, 49.47 ± 4.01%, and 28.27 ± 2.26% lower than that of the control group.

These data demonstrated that the EPS of *P. cornucopiae* SS-02 can increase antioxidant abilities by improving SOD, GSH-Px and CAT activities, and reducing MDA content in heart, liver, spleen, kidney and blood of mice. The mechanism by which the activity of these enzyme is increased and by which MDA production is inhibited could be direct activation of the enzymes or binding of metal ions which are necessary for organisms to produce free radical (Volpi and Tarugi, 1999; Gülçin et al., 2005).

Conclusion

In conclusion, a three-factor-three-level CCD design is a successful tool for extraction optimization of EPS by *P. cornucopiae* SS-02 in submerged culture. The EPS showed antioxidant activities in vitro and in vivo. The results provide a reference for large-scale extraction of EPS by *P. cornucopiae* SS-02 in industrial fermentation and the EPS can be used as a potential antioxidant which

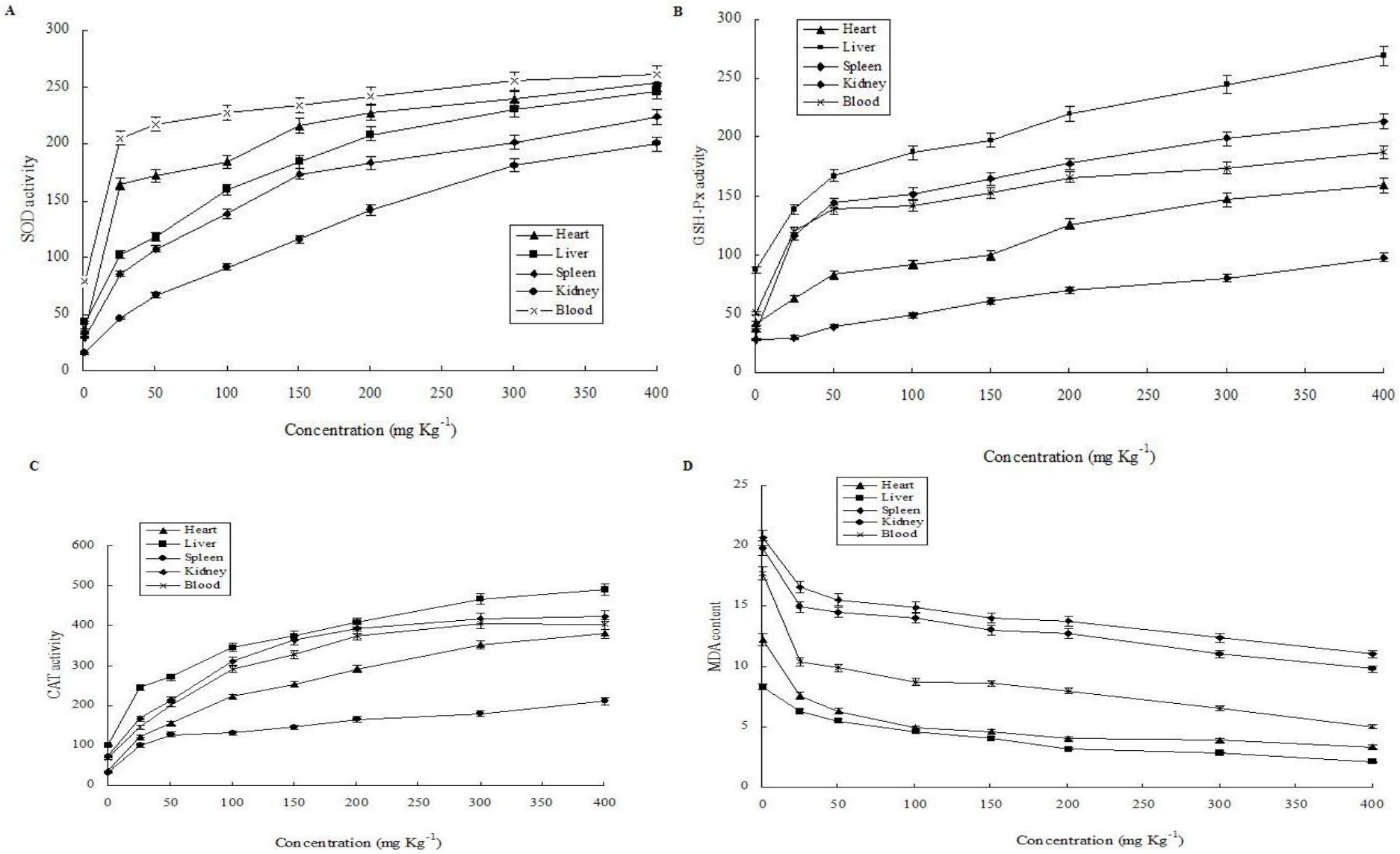

Figure 3. Effects of EPS on biochemical parameters in blood, liver, heart, spleen, and kidney of mice. (A) SOD activity, (B) GSH-Px activity, (C) CAT activity, and (D) MDA content. The units of SOD, GSH-Px and CAT activity are expressed as U/mg protein in liver, heart, spleen, and kidney, and U/ml in blood. The unit of MDA content is expressed as nmol/mg protein in liver, heart, spleen, and kidney, and nmol/ml in blood.

enhances adaptive immune responses.

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