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Extraction optimization and bioactivity of exopolysaccharides from *Agaricus bisporus*

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ABSTRACT

Response surface methodology was used to optimize the extraction parameters of EPS produced by *Agaricus bisporus* MJ-0811 in submerged culture. The optimal levels for ethanol concentration (85%, v/v), pH (8) and precipitation time (22 h) were determined, and EPS production was estimated at 2.71 g/L. The actual yield of EPS under these conditions was 2.69 g/L. In addition, the antioxidant activity of EPS was investigated by measuring its scavenging ability on superoxide radicals and hydroxyl radicals *in vitro*. Furthermore, the hypoglycemic activity of EPS was investigated by measuring its effects on body weights and blood glucose of diabetic mice. The study suggests that EPS has beneficial antioxidant activities (hydroxyl radical-scavenging activities, superoxide radical-scavenging activities) *in vitro*, anti-diabetic effect on alloxan induced diabetic mice. The EPS from *A. bisporus* may be a novel resource of natural antioxidants and anti-diabetic agents for use in the functional food or medicine.

1. Introduction

In recent years, exopolysaccharides (EPS) which are produced during the metabolic process of microorganisms such as bacteria, fungi, and blue-green algae, have been used as an important origin of microbial polysaccharides (Cho, Oh, Chang, & Yun, 2006; Xiang, Xu, & Li, 2012; Ye et al., 2011). For example, Hwang et al. (2005) reported the hypoglycemic effect of crude exopolysaccharides produced by a medicinal mushroom *Phellinus baumii* in streptozotocin-induced diabetic rats. He et al. (2012) studied fermentation optimization, characterization and bioactivity of EPS from *Funalia trogii*. They found that EPS from *F. trogii* was effective in the therapy of free radical injury and cancer diseases. Smiderle et al. (2012) investigated the effect of different carbon sources on the yield and fungi nutrient composition in *Pleurotus pulmonarius* submerged culture. However, there is little information about EPS from *Agaricus bisporus*.

A. bisporus, commonly known as the white button mushroom (WBM), is one of the most economically important edible mushrooms, and contains rich proteins, polysaccharides, vitamins, nucleotides and unsaturated fatty acids (Cremades et al., 2012; Ruthes, Rattmann, Carbonero, Gorin, & Iacomini, 2012; Tian et al., 2012; Xie et al., 2011). Some researchers investigated the extraction of bioactive substance from the fruiting body, the common edible form of *A. bisporus* (Tian et al., 2012; Wu & Ban, 2011; Xie et al., 2012). Furthermore, there are many evidences about the biological activities (antioxidant, hypoglycemic, hypolipidemic, anti-tumor, and so on) of *A. bisporus* (Jeong et al., 2010; Komura et al., 2010; Kozarski et al., 2011; Smiderle et al., 2011).

Until now, the optimal parameters of EPS extraction from *A. bisporus* in submerged culture, antioxidant and hypoglycemic potential have not been reported. Based on previous work, three significant factors (precipitation time, ethanol concentration and pH) affecting the extraction of EPS were chosen to optimize the extraction technology using response surface methodology (RSM) (Wu, Cui, Tang, & Gu, 2007; Xu Jie & Wei, 2008). The antioxidant and hypoglycemic potential (Cui et al., 2012; Kozarski et al., 2012; Li et al., 2006; Mao et al., 2009; Raza et al., 2012) of EPS from *A. bisporus* in submerged culture were also investigated in this work.

2. Materials and methods

2.1. Materials and reagents

A. bisporus MJ-0811 was obtained from school of biotechnology, Jiangnan University, China and maintained on synthetic potato dextrose agar (PDA). Glucose standard, potassium dihydrogen phosphate (KH_2PO_4), magnesium sulfate ($MgSO_4 \cdot 7H_2O$), peptone and agar were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China); other reagents are of analytical grade. Male

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Table 1
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	20	28
Ethanol concentration (%, v/v)	X_2	X_2	70	80	90
pH	X_3	X_3	7	8	9

mice were purchased from Experimental Animal Center of Soochow University.

2.2. Culture conditions

The strain of *A. bisporus* MJ-0811 was incubated in a PDA culture medium for activation of 4 days at 30 °C, and stored as the seeds for shake-flask fermentation. Cultivation in liquid media was carried out in 250 mL triangular flask containing 50 mL of (g/L): peptone, 2; corn steep liquor, 15; glucose, 20; KH_2PO_4, 2; and $MgSO_4{\cdot}7H_2O$, 1 with natural pH. Flasks were inoculated with a 0.5 cm^2 mycelial block of *A. bisporus* MJ-0811 from the solid media, and then shook on a rotary shaker at 150 rpm for 4 days.

2.3. Extraction and determination of A. bisporus EPS

A. bisporus cultures were centrifuged at 6000 rpm for 10 min. Then, the supernatant was mixed with three volumes of 95% ethanol (v/v), stirred vigorously and kept at 4 °C for 24 h. After centrifugation (6000 rpm, 10 min), the supernatant was removed; the sediment was dissolved with distilled water (60 °C). The solution was concentrated in a rotary evaporator at 50 °C and removed protein layer using method of Sevage. Crude EPS were obtained after freeze-drying and applied to detect antioxidant activities *in vitro* and hypoglycemic activity.

EPS content was determined by the phenol–sulfuric acid method using glucose as the standard and the protein content was determined using the Kjeldahl method with a conversion factor of 6.25 (Wu et al., 2007).

2.4. Response surface optimization for EPS extraction

Based on previous single-factor experiments, three independent variables (X_1, precipitation time; X_2, ethanol concentration; and X_3, pH) at three levels were adopted. Triplicates at the center (0, 0 and 0) of the design were conducted to allow the estimation of the pure error sum of squares. For statistical calculation, the variables were coded according to the following equation:

$$X_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

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 on the center point and ΔX_i is the step change value. As shown in Table 1, these three independent variables were coded as X_1, X_2, and X_3, respectively.

The actual design of experiments is shown in Table 2, and the behavior of the system explained by the following second-degree polynomial equation:

$$Y = b_0 + \sum b_i X_i + b_{ii} {X_i}^2 + b_{ij} X_i X_j \quad (2)$$

where Y is the predicted response value, b_0 is the intercept term, b_i is the linear term, b_{ii} is the squared term, b_{ij} is the interaction term, X_i and X_j are the coded level of independent variables.

Table 2
Experimental and predicted values of EPS based on Box–Behnken design.

Run	Variables			EPS content (g/L)	
	Coded			Experimental	Predicted
	X_1	X_2	X_3	Y_{Exp}	Y_{Pre}
1	−1	−1	0	1.46	1.38
2	−1	1	0	1.67	1.70
3	1	−1	0	2.07	2.04
4	1	1	0	2.36	2.44
5	0	−1	−1	1.38	1.42
6	0	−1	1	1.99	1.92
7	0	1	−1	1.82	1.89
8	0	1	1	2.13	2.09
9	−1	0	−1	1.10	1.14
10	1	0	−1	2.23	2.22
11	−1	0	1	1.83	1.84
12	1	0	1	2.21	2.17
13	0	0	0	2.60	2.60
14	0	0	0	2.62	2.60
15	0	0	0	2.57	2.60

2.5. Hypoglycemic experiments

Male mice (25 ± 3 g) were kept for environmental adaptation for one week before the experiment. After being weighed and fasted 12 h, forty mice were intraperitoneally injected with freshly prepared 2% alloxan (200 mg/kg). Seventy-two hours after alloxan injection, whole blood samples were obtained from the tail vein of the overnight fasted mice for the determination of fasting blood glucose with glucometer (Roche Diagnostics Ltd.). The mice with blood-glucose value above11.38 mmol/L were regarded as the diabetic mice model.

After establishment of alloxan diabetic mice model, the mice were divided into four groups: NM, norm-glycemic untreated mice receiving saline by oral administration; DM, diabetic mice receiving saline by oral administration; DM + Mnh, diabetic mice receiving metformin hydrochloride (200 mg/kg bw) by oral administration; DM + LEPS, diabetic mice receiving EPS from *A. bisporus* in submerged culture at the low dosage (200 mg/kg bw) by oral administration; DM + HEPS, diabetic mice receiving EPS from *A. bisporus* in submerged culture at the high dosage (400 mg/kg bw) by oral administration.

2.6. Antioxidant activities of EPS in vitro

2.6.1. Scavenging activity of hydroxyl radicals

The hydroxyl radical scavenging activity of the EPS was determined according to the method described by Ye, Liu, Wang, Wang, and Zhang (2012) with some modification. The reaction mixture contained 1.0 mL phosphate buffer saline (pH 7.4, 0.15 mM), 0.2 mL safranine T (0.26 mg/mL), 1.0 mL EDTA–Fe (II) (6 mM), 0.8 mL H_2O_2 (6%, v/v) and 7.0 mL EPS sample solution, and the mixture was incubated at 40 °C for 30 min. The absorbance of EPS was measured immediately at 520 nm, using Vc as a positive control. The scavenging ability was calculated according to the following equation:

$$\text{Scavenging ability}\,(\%) = \frac{A_s - A_0}{A - A_0} \quad (3)$$

where A_s is the absorbance of the reagent mixture with the sample, A_0 is the absorbance of the reagent mixture without the sample, and A is the absorbance of the reagent mixture without the sample and H_2O_2.

2.6.2. Scavenging activity of superoxide anion

The scavenging ability of superoxide anion was measured according to the method reported by Chen et al. (2012). 3 mL of Tris–HCl buffer (0.05 M, pH 8.2) was mixed with 1 mL EPS

Table 3
Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of EPS.

Source	Sum of squares	df	Mean of square	*F* value	*p*-Value
Model	2.94	9	0.33	52.84	0.0002
X_1	0.25	1	0.25	40.73	0.0014
X_2	0.99	1	0.99	159.5	<0.0001
X_3	0.21	1	0.21	33.61	0.0022
X_1X_2	0.0016	1	0.0016	0.26	0.6328
X_1X_3	0.022	1	0.022	3.64	0.1149
X_2X_3	0.14	1	0.14	22.72	0.005
X_1^2	0.48	1	0.48	77.15	0.0003
X_2^2	0.44	1	0.44	71.88	0.0004
X_3^2	0.61	1	0.61	98.88	0.0002
Residual	0.031	5	0.006		
Lack of fit	0.03	3	0.099	15.62	0.0608
Pure error	0.0013	2	0.00061		
Cor total	2.97	14			

solution. After incubation at 25 °C for 10 min, 200 μL of pyrogallol at the same temperature were added to the mixture, and the reaction kept at 25 °C for 4 min. Finally, the reaction system was terminated by the addition of 0.5 mL of HCl. The absorbance of EPS was measured immediately at 320 nm. The scavenging ability was calculated according to the following equation:

$$\text{Scavenging ability } (\%) = 1 - \frac{A_S}{A} \quad (4)$$

where A_S is the absorbance of the reagent mixture with the sample and A is the absorbance of the reagent mixture without the sample.

2.7. Statistical analysis

The results were analyzed by one-way analysis of variance (ANOVA) test using the Statistical Package of the Social Science (SPSS) program. All results were expressed as the mean ± SD ($n = 10$). Differences were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Model fitting and statistical analysis

The experimental and predictive values of responses (extraction yield of EPS) under different treatment conditions are presented in Table 3. The predicted response Y_{Pre} for EPS was based on the following second-order polynomial equation:

$$Y_{Pre} = 2.60 + 0.18X_1 + 0.35X_2 + 0.16X_3 + 0.022X_1X_2 - 0.076X_1X_3 - 0.18X_2X_3 - 0.36X_1X_1 - 0.34X_2X_2 - 0.41X_3X_3 \quad (5)$$

The model can be proved fit, and to adequately account for the variation observed if the *F*-test for the model is significant at $p < 0.05$ (Li, Xu, Li, Feng, & Ouyang, 2012). The smaller *p*-value indicated the corresponding variable was more significant. As showed in Table 2, the model was significant with an *F*-test of a very low probability value ($p > F$) < 0.0001. On three variables (X_1, X_2, X_3) and X_2X_3, the *p*-values were much less than 0.01, indicating that all these variables were more significant. The fit of the model is indicated by the determination coefficient (R^2). The closer the value of R^2 to the 1, the better the correlation between experimental and predicted data (Wu et al., 2007). The value of R^2 for Eq. (5) was 0.9896, suggesting that 98.96% of the total variables could be predicted by the model. The value of adj-R^2 (0.9702) suggested that the total variation of 97.02% for the EPS extraction was attributable to the independent variables and only about 2.98% of the total variation could not be explained by the model.

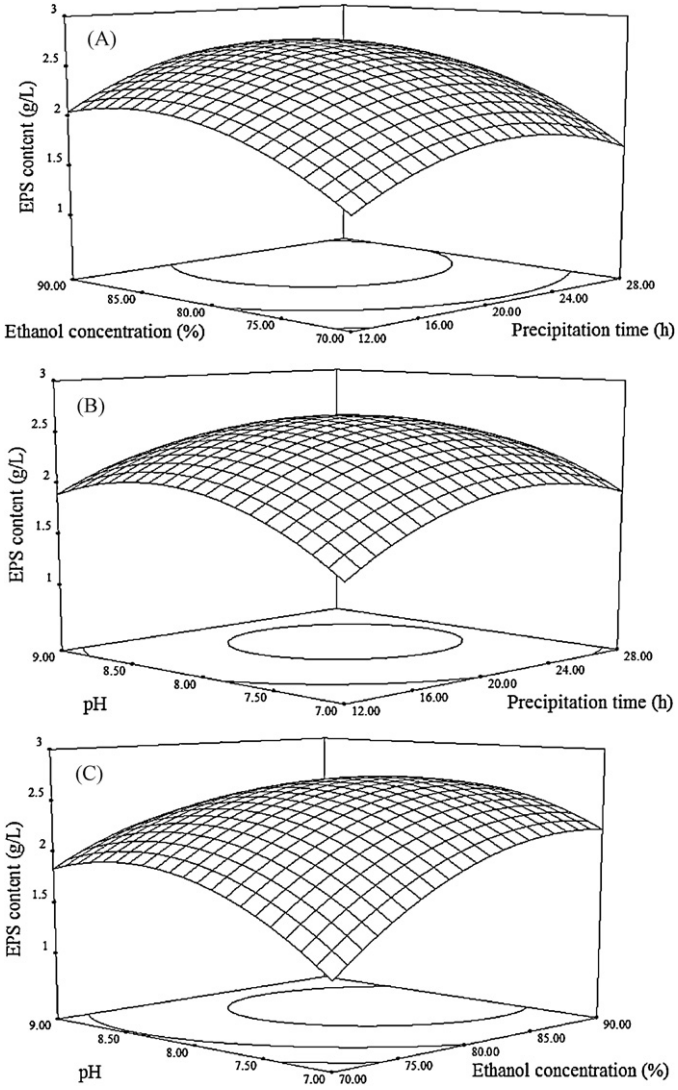

Fig. 1. Response surface 3D plots for the yield of EPS from fermentation broth in submerged culture of *Agaricus bisporus* in terms of the effects of (A) precipitation time and ethanol concentration, (B) pH and precipitation time, and (C) pH and ethanol concentration. Factors that were not included in the axes were fixed at their respective optimum levels.

3.2. Analysis of response surfaces

The graphical representations of the regression Eq. (5), called the response surfaces and the contour plots, are presented in Fig. 1. These 3D response surface and contour plots provide a visual interpretation of the interactions between two variables with one variable kept at zero levels and ease the location of optimum experimental conditions. Fig. 1(A) shows the effect of ethanol concentration and precipitation time on EPS extraction. A quadratic effect of ethanol concentration and precipitation time on the response was observed. Fig. 1(B) shows the effect of pH and precipitation time on EPS extraction. A quadratic effect of pH and precipitation time on the response was observed. Fig. 1(C) shows the effect of ethanol concentration and pH on EPS extraction. A quadratic effect of ethanol concentration and pH on the response was observed.

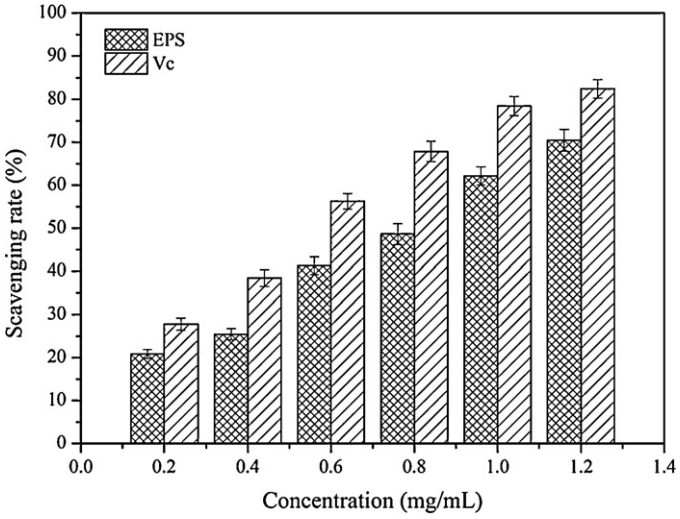

Fig. 2. Scavenging effect of EPS on superoxide anion radicals. Data are the means of three replicates with standards deviations shown by vertical bars.

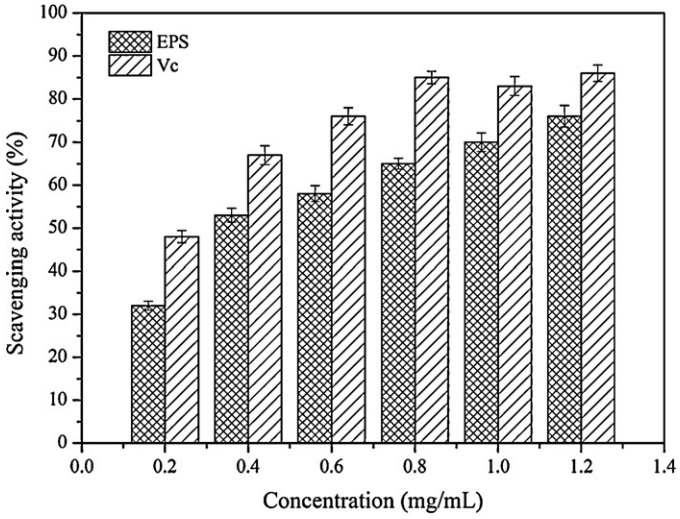

Fig. 3. Scavenging effect of EPS on hydroxyl radicals. Data are the means of three replicates with standards deviations shown by vertical bars.

3.3. Optimization of extraction parameters and validation of the model

According to the regression equation, the predicted maximum value of EPS extraction was 2.71 g/L when ethanol concentration is 85.05%, pH is 8.06 and precipitation time is 22.03 h. In view of the operating convenience, the optimal parameters were determined to be ethanol concentration 85% (v/v), pH 8.0 and precipitation time 22 h. Based on these optimal parameters, the predicted value of EPS extraction was 2.69 g/L, which was slightly lower than the predicted maximum value.

In order to ensure the suitability of the model equation for predicting the optimum response values, experimental rechecking was performed using the recommended optimum conditions. It was found that the experimental value (2.66 ± 0.65 g/L, $n = 3$) was in agreement with the predicted one, indicating that the response surface model was suitable for optimizing the EPS extraction process. The crude EPS extracted under the optimum conditions were further analyzed for compositions. The crude EPS contained 64.37% carbohydrates and 28.82% proteins.

3.4. Scavenging activity of hydroxyl radicals

Hydroxyl radicals, which are the most reactive among reactive oxygen species (ROS), can do great damage to intracellular biomacromolecule and lead to oxidation reaction of liposome (Tian et al., 2012). Some researchers have reported that the hydroxyl radicals scavenging activities of EPS were about 58.22% for *Pholiota adiposa* SX-01 at 250 mg/L (Sun, 2012), 63.64% for *Cordyceps militaris* SU5-08 at 5 g/L (Lin et al., 2012), 35% for *Lactococcus lactis* subsp. *lactis* 12 at 1.0 mg/mL (Pan & Mei, 2010), 92.12% for *Pseudomonas* PF-6 at 0.6 mg/mL (Ye et al., 2012), respectively. In this experiment, hydroxyl radicals were generated through the Fenton reaction. Thus, the hydroxyl radicals scavenging activity was figured out based on the absorbance reduction of the reaction system. Fig. 2 shows the hydroxyl radicals scavenging activity of EPS from *A. bisporus* with Vc as positive control. The scavenging effects were generally enhanced with the increase of concentration. The hydroxyl radicals scavenging activity of EPS at 1.2 mg/mL reached 76%, which was not significantly lower than those of ascorbic acid. The results indicated that EPS from *A. bisporus* had good scavenging activity on hydroxyl radicals; however, this requires further investigations.

3.5. Scavenging activity of superoxide anion

Superoxide radicals are generated by natural metabolizable action and oxygen activation reaction. They are considered as an initial free radical, to create secondary active oxygen, such as hydroxyl radical, H_2O_2, or singlet oxygen (Xie et al., 2012). Superoxide radicals and other secondary active oxygen can do great damage to intracellular lipid, protein and DNA (Raza, Makeen, Wang, Xu, & Qirong, 2011). Thus, superoxide radical scavenging activities could be used an important antioxidant index *in vitro*. Ye et al. (2012) reported that the superoxide radicals scavenging activity of EPS from *Pseudomonas* PF-6 was 80% at 0.25 mg/mL. It was also found that the superoxide radical scavenging activity was about 70% for *Gomphidius rutilus* at 0.25 mg/mL (Gao, Wang, Su, Zhang, & Yang, 2012), 45% for *Morchella esculenta* SO-02 at 1.0 mg/mL (Meng et al., 2010), 47% *Paenibacillus polymyxa* SQR-21 at 1.0 mg/mL (Raza et al., 2011), *etc.* Fig. 3 shows the superoxide radicals scavenging activity of EPS from *A. bisporus* with Vc as positive control. The results indicated that the superoxide radicals scavenging activity of EPS was in a concentration-dependent manner, generally enhanced by the increase in concentrations. Superoxide radicals scavenging activity of EPS at 1.2 mg/mL reached 82.45% ($p < 0.01$). The results indicated that EPS of *A. bisporus* had beneficial scavenging activity on hydroxyl radicals.

3.6. Hypoglycemic activity of EPS

Diabetes mellitus has become a major health problem, affecting approximately 3% of the population worldwide (Tong, Liang, & Wang, 2008). However, as clinically main measure, the medical treatment and management of diabetes mellitus have a number of side effects. Thus, it is necessary to develop alternative therapies for diabetes mellitus without troublesome side. In order to evaluate the hypoglycemic activity of EPS, alloxan diabetic mice which are prevalent animal diabetic model used in hypoglycemic experiments were used in this experiment. The effects of EPS on blood glucose and body weight are shown in Table 4. When compared to control (non-diabetic mice), blood glucose increased and body weight decreased noticeably in diabetic mice. After treatment of EPS in diabetic mice for 14 days, there was a significant ($p < 0.01$) increase in body weight and a significant ($p < 0.01$) decrease in blood glucose. For example, after the continuous administration for 14 days with the dosage of 200 mg/kg bw of EPS, the blood glucose

Table 4
Effect of EPS on body weight and blood glucose in diabetic mice.

Groups	Dosage (mg/kg bw)	Body weight (g/mice)		Blood glucose (mmol/L)	
		0 days	14 days	0 days	14 days
NM	0	24.56 ± 2.45	28.82 ± 2.17	5.87 ± 0.58	5.68 ± 0.67
DM	0	19.37 ± 1.78[a]	18.25 ± 2.12[a]	22.37 ± 1.73[a]	23.45 ± 1.52[a]
DM + LEPS	200	19.82 ± 2.52[a]	21.35 ± 1.96[a,b,c]	22.12 ± 1.45[a]	15.34 ± 1.67[a,b,c]
DM + HEPS	400	19.45 ± 1.37[a]	22.17 ± 2.16[a,b]	22.56 ± 2.16[a]	12.28 ± 1.67[a,b,c]
DM + Mnh	20	19.26 ± 1.69[a]	22.08 ± 1.53[a,b]	22.28 ± 1.86[a]	10.37 ± 2.13[a,b]

Values are expressed as mean ± SD for six mice in each group. One-way ANOVA repeated measures with Duncan's multiple rang test was used to calculate statistical significance. NM, norm-diabetic mice; DM, diabetic mice; DM + LEPS, diabetic mice receiving EPS from *Agaricus bisporus* in submerged culture at the low dosage (200 mg/kg bw) by oral administration; DM + HEPS, diabetic mice receiving EPS from *Agaricus bisporus* in submerged culture at the high dosage (400 mg/kg bw) by oral administration; DM + Mnh, diabetic mice receiving metformin hydrochloride (200 mg/kg bw) by oral administration.

[a] $p \leq 0.01$, significantly different from the NM group.
[b] $p \leq 0.01$, significantly different from the DM group.
[c] $p \leq 0.05$, significantly different from the metformin hydrochloride group.

in alloxan diabetic mice significantly reduced from 22.12 nmo/L to 15.34 nmol/L ($p < 0.01$); when the dosage of EPS was 400 mg/kg bw, the blood glucose in alloxan diabetic mice significantly ($p < 0.01$) reduced from 22.56 to 12.58 nmol/L; the blood glucose in metformin hydrochloride group decreased significantly ($p < 0.01$) when compared with diabetic mice. Body weights in EPS group increased significantly ($p < 0.01$) when compared with diabetic mice group, but the body weight in diabetic mice group decreased significantly ($p < 0.01$). The results indicated that the EPS from *A. bisporus* had a strong hypoglycemic effect on alloxan diabetic mice with dose-dependence.

4. Conclusion

Through response surface methodology, the optimal levels for ethanol concentration (85%, v/v), pH (8.0) and precipitation time (22 h) were determined. The experimental values identified under these conditions were closely correlated to the predicted ones. EPS showed potent antioxidant activities (hydroxyl radical-scavenging activities, superoxide radical-scavenging activities) *in vitro*. EPS also exerted a potent hypoglycemic effect. The results indicated that response surface methodology using second-order regression for a Box–Behnken design may be used as a productive tool for the optimization of extraction of EPS produced by *A. bisporus* MJ-0811 in submerged culture. Our results also suggested that EPS extracted from *A. bisporus* MJ-0811 was expected to be made full development and utilization of antioxidative activity and hypoglycemic activity for use in the functional food or medicine.

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