Carbohydrate Polymers 81 (2010) 533–540

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Medium optimization, preliminary characterization and antioxidant activity *in vivo* of mycelial polysaccharide from *Phellinus baumii* Pilát

Jianguang Luo[a], Jun Liu[a], Yi Sun[a], Hong Ye[a], Chunhong Zhou[b], Xiaoxiong Zeng[a,*]

[a] College of Food Science and Technology, Nanjing Agricultural University, Nanjing 210095, China
[b] Jiangsu Environmental Monitoring Center, Nanjing 210036, Jiangsu, China

ARTICLE INFO

Article history:
Received 4 February 2010
Received in revised form 2 March 2010
Accepted 2 March 2010
Available online 9 March 2010

Keywords:
Phellinus baumii Pilát
Mycelial polysaccharide
Medium components
Statistical experimental design
Antioxidant activity *in vivo*

ABSTRACT

Optimization of medium ingredients for the production of mycelial polysaccharides from *Phellinus baumii* Pilát (PBMP) in submerged culture, preliminary characterization and the evaluation of antioxidant activity *in vivo* of PBMP were carried out. An optimal medium for PBMP production was obtained through a $2^{(7-3)}$ fractional factorial design and a central composite design in response surface methodology as follows (g/l): glucose 35.36, yeast extract 2.88, peptone 2.73, $MgSO_4$ 1.47, KH_2PO_4 1, VB_1 0.0075 and diammonium oxalate monohydrate 0.3. The resulting PBMP was composed of D-glucose, D-galactose, L-fucose, D-mannose, and L-rhamnose in a molar ratio of 12.74:1.39:1.00:1.92:0.22. It was demonstrated that the administration of PBMP could increase the activities of antioxidant enzymes, decrease the levels of malondialodehyde, and enhance total antioxidant capabilities in livers and serums of D-galactose-induced mice. The results suggested that PBMP had potent antioxidant activity and could be explored as novel natural antioxidant.

1. Introduction

Phellinus baumii Pilát, a basidiomycete fungus belonging to the genus *Phellinus* in the family of *Hymenochaetaceae*, is a precious and highly acclaimed medicinal fungus in traditional Chinese medicine. The polysaccharides isolated from the fruiting body of *P. baumii* Pilát have been reported to have potential antioxidant, anti-inflammatory, immuno-stimulating, and antitumor activities (Chang et al., 2007; Ge, Zhang, & Sun, 2009; Jang et al., 2004; Shon, Kim, & Sung, 2003). In addition, the anti-diabetic effect and immuno-stimulating activity of exopolysaccharides from submerged mycelial culture of *P. baumii* Pilát have also been reported (Hwang et al., 2005; Luo et al., 2009). As we know, the cultivation of the fruiting body of *P. baumii* Pilát requires 5–6 months and its product quality is difficult to control when *P. baumii* Pilát is traditionally cultivated in solid culture. In contrast, submerged culture has potential advantages for higher mycelial and polysaccharide production in a more-compact space over a shorter incubation time and availability of convenient control with less chance of contamination (Kim et al., 2002; Lee et al., 2004). Therefore, submerged culture has become a promising alternative for efficient production of valuable metabolites, especially polysaccharides. Many investigators have attempted to obtain optimal submerged cultures for mycelial polysaccharides production from several fungi (Dong & Yao, 2005; Shih, Pan, & Hsieh, 2006), but to the best of our knowledge, the nutritional requirements for submerged culture of *P. baumii* Pilát have not been demonstrated. To achieve higher yield of mycelial polysaccharide in a submerged culture, it is a prerequisite to design an optimal production medium and a set of optimal process operating conditions.

Medium optimization by conventional techniques such as one-factor-at-a-time method involves changing one independent variable while fixing the others at certain levels. This single-dimensional search is laborious and time-consuming, and overlooks the interaction between different variables involved. Alternatively, the statistical experimental design such as factorial design and response surface techniques provides a number of potential advantages, for instance more advanced results with less process variability, closer confirmation, less development time and less overall costs. In fact, the fermentation processes of many medicinal fungi have been optimized by using this methodology to improve the production of primary and secondary metabolites (Chen, Zhao, Chen, & Li, 2008; Liu, Miao, Wen, & Sun, 2009; Luo et al., 2009; Mao, Eksriwong, Chauvatcharin, & Zhong, 2005). Therefore, the medium for the production of mycelial polysaccharide from *P. baumii* Pilát (PBMP) was optimized by using $2^{(7-3)}$ fractional factorial design (FFD) and central composite design (CCD) of response surface methodology (RSM) in the present study. In addition, the preliminary characterization and analysis of antioxidant activity *in vivo* for PBMP were carried out.

* Corresponding author. Fax: +86 25 84396791.
E-mail address: zengxx@njau.edu.cn (X. Zeng).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.03.010

2. Materials and methods

2.1. Microorganism

P. baumii Pilát used in this study was obtained from China Forestry Culture Collection Center (CFCC, Beijing, China). The stock culture was maintained on PDA slants supplemented with peptone 2 g/l, KH_2PO_4 1 g/l, and $MgSO_4$ 0.5 g/l. The slant was incubated at 28 °C for 10 days and then stored in a refrigerator (about 4 °C), and sub-cultured every 2 months.

2.2. Inoculum preparation and flask cultures

The seed culture was grown in a 250 ml Erlenmeyer flask containing 50 ml of basal medium (glucose 20 g/l, peptone 2 g/l, yeast extract 1 g/l, KH_2PO_4 1 g/l, $MgSO_4$ 0.5 g/l, thiamine (VB_1) 0.01 g/l, distilled water, initial pH 6.0) at 28 °C on a rotary incubator at 150 rpm for 7 days. Flask culture experiments were performed in 250 ml Erlenmeyer flasks containing 50 ml medium after inoculating with 10% (v/v) of the seed culture at 28 °C on a rotary shaker at 150 rpm for 6 days.

2.3. Preparation of PBMP

The mycelia in different cultural conditions were harvested by filtering through a filter paper to separate them from the liquid medium. After repeated washing of the mycelial pellets with distilled water and drying at 60 °C to a constant weight, the dry weight (DW) of the mycelia was determined in terms of g/l. Then, the dry mycelia were extracted 3 times with distilled water (the ratio of dry mycelia and distilled water was 1:20) at 90 °C for 2 h each, and centrifuged at 5000 rpm for 20 min. The supernatants were combined and concentrated to one-fifth of its original volume with a rotary evaporator under reduced pressure. The resulting residue was mixed with 3 times volume of absolute ethanol, stirred vigorously and kept overnight at 4 °C. The precipitate was collected by centrifugation, washed twice with acetone and ether, respectively, and dried to afford PBMP. The weight of PBMP was estimated in terms of g/l (DW).

2.4. Optimization procedure and experimental design

2.4.1. Identifying the significant variables using FFD

In order to determine the key ingredients significantly affecting the production of PBMP, FFD was employed for the screening of the important medium components with respect to their main effects as described previously (Luo et al., 2009). The seven nutrient factors chosen for the present study were glucose, peptone, yeast extract, diammonium oxalate monohydrate, KH_2PO_4, $MgSO_4$ and VB_1. As shown in Table 1, all the variables were denoted as numerical factors and examined at two widely spaced intervals at a high level (coded +1) and a low level (coded −1). A 1/8 fraction of the full factorial design was adopted and consequently the experiment included 16 (2^{7-3}) combinations plus four replicates at the center point. Each trial was carried out in duplicate and PBMP production was measured after 6 days of cultivation.

2.4.2. Central composite design

The levels of the significant parameters and interaction effects between various medium components which influence significantly the PBMP production were analyzed and optimized by using a CCD in RSM. The experimental design was carried out by using "Stat-Ease Design-Expert" software (version 7.1.3, Stat-Ease Corporation, USA). The four independent factors were investigated at five different levels (−2, −1, 0, +1, +2), and the experimental design is shown in Table 3. Briefly, the variables were coded according to Eq. (1):

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

where X_i is the real value of variable, X_0 the real value of the X_i at the center point, ΔX_i step change in X_i, x_i the coded value of the variable, and $i = 1, 2, 3$. The experimental levels for these variables were selected from our preliminary work, which indicated that

Table 1
The fractional factorial design for screening of significant factors affecting the production of mycelial polysaccharide from *P. baumii* Pilát (PBMP).

Run	Factors							Mycelial dry weight (g/l)	Response
	A[a]	B[b]	C[c]	D[d]	E[e]	F[f]	G[g]		PBMP (g/l)
1	0	0	0	0	0	0	0	6.62 ± 0.69	0.555 ± 0.01
2	−1	−1	+1	−1	+1	+1	+1	5.94 ± 0.14	0.509 ± 0.04
3	−1	+1	−1	+1	+1	−1	+1	8.23 ± 0.26	0.558 ± 0.07
4	+1	−1	+1	+1	−1	−1	+1	6.33 ± 0.41	0.487 ± 0.02
5	+1	+1	−1	−1	−1	+1	+1	5.67 ± 0.48	0.369 ± 0.03
6	−1	−1	−1	+1	−1	+1	+1	5.24 ± 0.37	0.297 ± 0.05
7	0	0	0	0	0	0	0	6.46 ± 0.47	0.575 ± 0.06
8	+1	+1	+1	−1	+1	−1	−1	9.40 ± 0.72	0.846 ± 0.06
9	+1	−1	−1	−1	+1	−1	+1	4.78 ± 0.86	0.238 ± 0.01
10	+1	+1	+1	+1	+1	+1	+1	9.52 ± 0.04	0.693 ± 0.01
11	−1	+1	+1	+1	−1	+1	−1	6.29 ± 0.98	0.421 ± 0.03
12	+1	+1	−1	+1	−1	−1	−1	6.95 ± 0.59	0.518 ± 0.03
13	+1	−1	+1	−1	−1	+1	−1	8.85 ± 0.63	0.562 ± 0.03
14	−1	−1	−1	−1	−1	−1	−1	6.48 ± 0.45	0.409 ± 0.03
15	−1	−1	+1	+1	+1	−1	−1	5.50 ± 0.24	0.466 ± 0.04
16	+1	−1	−1	+1	+1	+1	−1	4.69 ± 0.35	0.310 ± 0.03
17	−1	+1	−1	−1	+1	+1	−1	5.00 ± 0.43	0.348 ± 0.00
18	0	0	0	0	0	0	0	6.10 ± 0.73	0.504 ± 0.01
19	−1	+1	+1	−1	−1	−1	+1	4.67 ± 0.07	0.310 ± 0.00
20	0	0	0	0	0	0	0	7.31 ± 0.75	0.631 ± 0.03

[a] Glucose at a low level (−1) of 20 g/l and a high level (+1) of 40 g/l.
[b] Peptone at a low level (−1) of 2 g/l and a high level of 4 g/l.
[c] Yeast extract at a low level (−1) of 1 g/l and a high level of 3 g/l.
[d] KH_2PO_4 at a low level (−1) of 0.5 g/l and a high level of 1.5 g/l.
[e] $MgSO_4$ at a low level (−1) of 0.5 g/l and a high level of 1.5 g/l.
[f] Thiamine (VB_1) at a low level (−1) of 0.005 g/l and a high level of 0.01 g/l.
[g] Diammonium oxalate monohydrate at a low level (−1) of 0.1 g/l and a high level of 0.5 g/l.

an optimum value could be found within the level of parameters studied.

The response Y (yield of PBMP) was analyzed by using a second-order polynomial equation in four independent variables and the data were fitted into the equation by multiple regression procedure. The model equation for analysis is given below Eq. (2):

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j \quad (2)$$

where Y is the predicted response, X_i, X_j represent the independent variables which influence the response variable Y, and β_0, β_i, β_{ii}, and β_{ij} represent the offset term, the ith linear coefficient, the ith quadratic coefficient and the ijth interaction coefficient, respectively.

"Design-Expert" was used for regression and graphical analyses of the data obtained. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The Student's t-test permitted the checking of the statistical significance of the regression coefficient, and the Fischer's test determined the second-order model equation. The quality of the fit of the polynomial model equation was given by the coefficient of determination (R^2). The optimum concentrations of the variables were calculated from the data obtained by using the response surface regression procedure of the SAS statistical package (Version 8.1, SAS Institute Inc., NC, USA).

2.5. Preliminary characterization of PBMP

2.5.1. Determination of protein, carbohydrate and uronic acid contents

The contents of carbohydrate, protein, and uronic acid in PBMP were determined according to the reported methods (Blumenkrantz & Asboe-Hansen, 1973; Bradford, 1976; Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) by using D-glucose, bovine serum albumin, and D-glucuronic acid as standards, respectively.

2.5.2. Analysis of monosaccharide composition

For monosaccharide composition analysis by use of gas chromatography (GC), PBMP (5 mg) was hydrolyzed with 2 ml of 2 M trifluoroacetic acid at 120 °C for 2 h. The hydrolyzate was repeatedly co-distilled with methanol to dryness and conventionally converted into the aldonitrile acetates as described previously (Guerrant & Moss, 1984). The aldonitrile acetate derivatives of standard monosaccharides were prepared in the same way. Then, all the derivatives were analyzed on an Agilent 6890N GC equipped with flame ionization detector (FID) and an HP-5 fused silica capillary column (30 m × 0.32 mm × 0.25 mm). The following chromatographic conditions were used: nitrogen gas was used as the carrier gas at a flow rate of 1 ml/min; the temperature of injector and detector were set at 250 and 280 °C, respectively; the initial column temperature was held at 120 °C for 2 min, then programmed at a rate of 3 °C/min to 210 °C and held at 210 °C for 4 min.

2.5.3. Fourier-transform infrared spectrometric analysis

FT-IR spectrum was recorded on a Nicolet 6700 FT-IR spectrometer (Madison, WI, USA). The dried sample was grinded with potassium bromide (KBr) powder and pressed into pellet for spectrometric measurement at a frequency range of 4000–400 cm^{-1}.

2.6. Evaluation of antioxidant activity in vivo of PBMP

2.6.1. Animal grouping and experimental design

The antioxidant activity *in vivo* of PBMP was evaluated according to our reported method with some modifications (Ke et al., 2009; Qiao et al., 2009). Briefly, all experiments were performed with female Kunming mice (2-month old, body weight (BW) 20 ± 2 g), which were purchased from the Experimental Animal Center of Academy of the Military Medical Sciences (Beijing, China). The animals were maintained under controlled temperature (23 ± 0.5 °C), humidity (55 ± 5%) and light conditions (12-h light/12-h dark cycle). During the entire experimental period (including acclimation), all animals were allowed free access to standard laboratory pellet diet and water. All procedures involving animals were conducted in strict accordance with the Chinese legislation on the use and care of laboratory animals.

After a 7-day acclimation period, these mice were randomly assigned into six groups (six mice for each) including normal control group, D-galactose (D-Gal) model control group, vitamin C (Vc) group (as positive control), PBMP group (100, 200, 400 mg/kg BW). Mice in normal control group were given physiological saline (10 ml/kg BW) once daily by hypodermic injection and gastric gavage. Mice in model control group were fed D-Gal (100 mg/kg BW) by hypodermic injection and the same volume of physiological saline by gastric gavage once daily. Mice in Vc group were treated with D-Gal (100 mg/kg BW) by hypodermic injection and Vc (100 mg/kg BW) by gastric gavage once daily. Mice in PBMP groups were respectively fed with PBMP in three different doses (100, 200 and 400 mg/kg BW per day) by gastric gavage and D-Gal (100 mg/kg BW per day) by hypodermic injection. All groups were performed once daily for 40 consecutive days.

2.6.2. Biochemical assay

After overnight fasting following the last drug administration, the mice were weighed and killed by decapitation. Blood samples were collected immediately and centrifuged at 4000 × g at 4 °C for 10 min to afford the serums. The liver was excised, weighed and homogenized immediately in 0.1 g/ml wet weight of ice-cold physiological saline. The suspension was centrifuged and the supernatant was collected for further analysis. All above treatments were done at 4 °C.

The activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), protein content, malondialdehyde (MDA) level and total antioxidant capacity (TAOC) were determined by using commercially available kits (Nanjing Jiancheng Bioengineering Institute, China) according to the kits instructions. In brief, the protein content was measured according to the Bradford method using bovine serum albumin as the standard. The activities of SOD, CAT and GSH-Px were analyzed by the methods of xanthine oxidase–xanthine reaction system, CAT–H_2O_2 reaction system, reduced glutathione GSH–H_2O_2 reaction system, respectively. MDA level was analyzed with 2-thiobarbituric acid (TBA) method, and TAOC was measured by the method of ferric reducing/antioxidant power reaction system.

2.7. Statistical analysis

The Student's t-test for statistical analysis of data was used. Unless otherwise stated, all data in the text and figures are presented as means ± standard deviation (SD). Differences are considered significant when $p < 0.05$.

3. Results and discussions

3.1. Screening of parameters using FFD

FFD is a powerful method for screening significant factors. In the present study, 20 runs were carried out to investigate the effects of the relative significance of seven nutrition factors including glucose, peptone, yeast extract, diammonium oxalate monohydrate, KH_2PO_4, $MgSO_4$, and VB_1. Notably, the PBMP yield and mycelial dry weight (MDW) varied greatly from 0.238 to 0.846 and 4.67 to 9.52 g/l, respectively, under different combinations of the media components (Table 1). Furthermore, statistical analysis of

Table 2
Statistical analysis of fractional factorial design for screening the significant variables.

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	Prob > *F*
Model	0.38	14	0.027	12.94	0.0120[a]
A	0.031	1	0.031	15.03	0.0179[a]
B	0.039	1	0.039	18.59	0.0125[a]
C	0.097	1	0.097	47.00	0.0024[a]
D	1.603E−003	1	1.603E−003	0.77	0.4290
E	0.022	1	0.022	10.65	0.0310[a]
F	6.548E−003	1	6.548E−003	3.16	0.1503
G	0.011	1	0.011	5.30	0.0828
AB	0.047	1	0.047	22.88	0.0088
AC	0.070	1	0.070	33.83	0.0044
AD	1.855E−003	1	1.855E−003	0.89	0.3979
AE	5.327E−003	1	5.327E−003	2.57	0.1843
AG	0.014	1	0.014	6.92	0.0581
BD	0.014	1	0.014	6.79	0.0597
ABD	0.014	1	0.014	6.81	0.0594
Curvature	0.037	1	0.037	17.77	0.0135[a]
Residual	8.297E−003	4	2.074E−003		
Lack of fit	1.048E−006	1	1.048E−006	3.795E−003	0.9548[b]
Pure error	8.286E−003	3	2.762E−003		
Cor total	0.42	19			

R^2 (predict) = 0.9547 and R^2 (adjust) = 0.9028.
[a] 5% significance level.
[b] Not significant relative to the pure error due to noise.

the responses and the main effects of the selected variables were done and the results are shown in Table 2. The *F*- and *p*-value for the model were found to be 12.94 and 0.0120, respectively, which implied that the model was significant. The determination coefficient R^2 of the model was 0.9547, indicating that 95.47% of the variability in the response could be explained by the model. The "Curvature *p*-value" of 0.0135 implied there was significant curvature (as measured by difference between the average of the center points and the average of the factorial points) in the design space and the optimum value was inside the experimental design. The results showed that four variables viz. glucose, yeast extract, peptone and $MgSO_4$ significantly influenced the PBMP production ($p < 0.05$), while KH_2PO_4, VB_1 and diammonium oxalate monohydrate did not result in significant variation at 5% level for PBMP production. Thus, the significant variables including glucose (X_1), yeast extract (X_2), peptone (X_3) and $MgSO_4$ (X_4) were selected for further optimization.

3.2. Central composite design and response surface analysis

According to the results of FFD, the four key factors and their central points chosen for experimental designs of CCD were glu-

Table 3
Coded levels and real values (in the parentheses, g/l) for the experimental design and results of CCD for the production of mycelial polysaccharide from *P. baumii* Pilát (PBMP).

Run	Coded level and actual values				Mycelial dry weight (g/l)	Response (*Y*)
	X_1: glucose	X_2: yeast extract	X_3: peptone	X_4: $MgSO_4$		PBMP (g/l)
1	−1(20)	+1(3)	+1(4)	+1(1.5)	8.97 ± 0.23	0.598 ± 0.03
2	0(30)	0(2)	−2(1)	0(1)	11.91 ± 0.60	0.567 ± 0.04
3	0(30)	+2(4)	0(3)	0(1)	11.84 ± 0.14	0.774 ± 0.17
4	0(30)	0(2)	0(3)	0(1)	13.66 ± 0.21	0.869 ± 0.02
5	+1(40)	+1(3)	+1(4)	+1(1.5)	12.10 ± 0.58	0.802 ± 0.11
6	0(30)	0(2)	0(3)	0(1)	13.50 ± 0.66	0.812 ± 0.13
7	0(30)	0(2)	+2(5)	0(1)	11.23 ± 0.12	0.529 ± 0.03
8	−1(20)	−1(1)	−1(2)	−1(0.5)	8.79 ± 0.39	0.591 ± 0.05
9	+1(40)	−1(1)	−1(2)	−1(0.5)	10.34 ± 0.36	0.530 ± 0.03
10	0(30)	−2(0)	0(3)	0(1)	6.71 ± 0.93	0.521 ± 0.06
11	−2(10)	0(2)	0(3)	0(1)	7.12 ± 0.01	0.357 ± 0.00
12	+1(40)	−1(1)	−1(2)	+1(1.5)	13.91 ± 0.83	0.699 ± 0.17
13	0(30)	0(2)	0(3)	0(1)	10.60 ± 0.37	0.854 ± 0.05
14	0(30)	0(2)	0(3)	+2(2)	8.80 ± 0.16	0.740 ± 0.00
15	+1(40)	−1(1)	+1(4)	+1(1.5)	12.57 ± 0.21	0.494 ± 0.01
16	−1(20)	−1(1)	+1(4)	+1(1.5)	9.09 ± 0.21	0.619 ± 0.00
17	−1(20)	+1(3)	−1(2)	−1(0.5)	8.76 ± 0.20	0.346 ± 0.07
18	0(30)	0(2)	0(3)	−2(0)	10.95 ± 0.18	0.636 ± 0.06
19	+1(40)	+1(3)	−1(2)	+1(1.5)	11.40 ± 0.04	0.854 ± 0.03
20	+1(40)	+1(3)	+1(4)	−1(0.5)	10.48 ± 0.25	0.679 ± 0.02
21	+2(50)	0(2)	0(3)	0(1)	10.84 ± 0.60	0.509 ± 0.14
22	+1(40)	+1(3)	−1(2)	−1(0.5)	10.00 ± 0.11	0.693 ± 0.04
23	+1(40)	−1(1)	+1(4)	−1(0.5)	9.24 ± 0.79	0.572 ± 0.02
24	0(30)	0(2)	0(3)	0(1)	12.19 ± 0.72	0.761 ± 0.03
25	−1(20)	+1(3)	+1(4)	−1(0.5)	10.34 ± 0.18	0.631 ± 0.06
26	−1(20)	−1(1)	−1(2)	+1(1.5)	8.97 ± 0.27	0.564 ± 0.09
27	0(30)	0(2)	0(3)	0(1)	10.33 ± 0.03	0.825 ± 0.09
28	−1(20)	+1(3)	−1(2)	+1(1.5)	7.42 ± 0.56	0.570 ± 0.04
29	0(30)	0(2)	0(3)	0(1)	12.48 ± 0.50	0.801 ± 0.16
30	−1(20)	−1(1)	+1(4)	−1(0.5)	9.28 ± 0.77	0.621 ± 0.09

Table 4
Variance analysis of response surface quadratic model for mycelial polysaccharide production from *P. baumii* Pilát.

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*p*-value
Model	0.54	14	0.039	14.48	<0.0001[a]
X_1	0.051	1	0.051	19.15	0.0005[a]
X_2	0.042	1	0.042	15.86	0.0012[a]
X_3	5.341E−004	1	5.341E−004	0.20	0.6605
X_4	0.022		0.022	8.25	0.0116 [a]
X_1X_2	0.062	1	0.062	23.45	0.0002 [a]
X_1X_3	0.023	1	0.023	8.70	0.0099[a]
X_1X_4	2.355E−003	1	2.355E−003	0.89	0.3617
X_2X_3	7.370E−003	1	7.370E−003	2.77	0.1167
X_2X_4	9.774E−003	1	9.774E−003	3.67	0.0745
X_3X_4	0.018	1	0.018	6.76	0.0201[a]
X_1^2	0.22	1	0.22	84.29	<0.0001[a]
X_2^2	0.037	1	0.037	13.88	0.0020[a]
X_3^2	0.10	1	0.10	39.17	<0.0001[a]
X_4^2	0.020	1	0.020	7.33	0.0162[a]
Residual	0.40	15	2.660E−003		
Lack of Fit	0.032	10	3.247E−003	2.18	0.2010[b]
Pure error	7.433E−0003	5	1.487E−0003		
Cor total	0.58	29			

R^2 (predict) = 0.9311 and R^2 (adjust) = 0.8668.
[a] 5% significance level.
[b] Not significant relative to the pure error due to noise.

cose (X_1) 30 g/l, yeast extract (X_2) 2 g/l, peptone (X_3) 3 g/l and $MgSO_4$ (X_4) 1 g/l, respectively; whereas the other factors including KH_2PO_4, VB_1 and diammonium oxalate monohydrate were set at their middle levels of 1, 1, 0.0075, and 0.3 g/l, respectively. Table 3 showed the CCD and the corresponding experimental data. The experimental results were analyzed by multiple regression analysis and the following second-order polynomial equation was established to explain PBMP production:

$$Y = 0.820 + 0.046X_1 + 0.042X_2 + 4.718\text{E} - 003X_3 + 0.030X_4 + 0.062X_1X_2 - 0.038X_1X_3 + 0.012X_1X_4 + 0.021X_2X_3 + 0.025X_2X_4 - 0.034X_3X_4 - 0.090X_1^2 - 0.037X_2^2 - 0.062X_3^2 - 0.027X_4^2 \quad (3)$$

where Y represents PBMP yield (g/l); X_1, X_2, X_3 and X_4 represent the concentrations of glucose, yeast extract, peptone and $MgSO_4$, respectively.

The *F*-test for ANOVA was conducted to test the significance of Eq. (3) for the experimental data as shown in Table 4. The model *F*-value of 14.48 implied that the model was significant ($p < 0.0001$), indicating that there was only a 0.01% chance that the model *F*-value could occur due to noise. The fitness of the model was examined by determination coefficient R^2, which was calculated to be 0.9311, indicating that 93.11% of the variability in the response could be explained by the model. In such case, the linear model terms (X_1, X_2 and X_4), quadratic model terms (X_1^2, X_2^2, X_3^2 and X_4^2) and interactive model terms (X_1X_2, X_1X_3 and X_3X_4) were significant ($p < 0.05$). Additionally, the value of lack of fit for regression Eq. (3) was not significant ($p > 0.05$), indicating that the model equation was adequate for predicting PBMP production under any combination of values of the variables.

The interactions and optimal levels of the variables were determined by plotting the response surface curves. The response surfaces are inverted paraboloids and can be used to predict PBMP yields for different values of the test variables and to identify the major interactions between the test variables from the circular or elliptical nature of contour (Muralidhar, Chirumamila, Marchant, & Nigam, 2001). Response surface plots and contour plots are shown in Fig. 1, which depict the interactions between two variables by keeping the other variables at their zero levels for PBMP production. It is evident that the respective contour plots in 3D-response surface graphs provided a visual interpretation of the interaction between two factors and facilitated the location of optimum experimental conditions. Among the tested variables, the interactions of glucose and yeast extract, glucose and peptone, peptone and $MgSO_4$ were significant. However, the interactions between glucose and $MgSO_4$, yeast extract and peptone, yeast extract and $MgSO_4$ were not significant at 5% level. The model predicted the optimal values of X_1, X_2, X_3 and X_4 in the coded units as 0.54, 0.89, −0.26 and 0.93, respectively. Correspondingly, their actual values were 35.36, 2.88, 2.73 and 1.47 g/l for glucose, yeast extract, peptone and $MgSO_4$, respectively. Under the optimum conditions, the maximum predicted value of PBMP yield obtained was 0.91 g/l. When the fungus was grown in the optimized medium (glucose 35.36 g/l, yeast extract 2.88 g/l, peptone 2.73 g/l, $MgSO_4$ 1.47 g/l, KH_2PO_4 1 g/l, VB_1 0.0075 g/l and diammonium oxalate monohydrate 0.3 g/l), the observed PBMP production and MDW were 0.927 ± 0.04 g/l and 13.74 ± 0.07 g/l, respectively, which corresponded fairly well to the predicted values. Therefore, the present work has also proved that response surface methodology could be used as a valuable and dependable tool for the optimization of mycelial polysaccharide production from *P. baumii* Pilát in general.

3.3. Preliminary characterization of PBMP

With the optimum condition for the production of PBMP, the mycelia from the submerged culture of *P. baumii* Pilát were extracted with distilled water, and the resulting extract was precipitated with ethanol to afford PBMP with a yield of 7.03%. Then, the contents of total carbohydrate, protein, and uronic acid in PBMP were estimated to be 61.82, 1.15, and 1.70%, respectively. As the monosaccharide composition of PBMP, we found through GC analysis that it was composed of D-glucose, D-galactose, L-fucose, D-mannose, and L-rhamnose in a molar ratio of 12.74:1.39:1.00:1.92:0.22. In addition, PBMP was characterized by FT-IR spectroscopy as shown in Fig. 2. The strong bands at 3368.80 and 2934.07 cm^{-1} were due to the hydroxyl stretching vibration and C–H stretching vibration of the polysaccharide, respectively. The band at 1654.87 cm^{-1} was due to the bound water. The three stretching vibration peaks between 1151.97 and 1024.56 cm^{-1} in the IR spectrum suggested the presences of C–O–C and C–O–H link bonds positions (Zhao, Yang, Yang, Jiang, & Zhang, 2007). In addition, the bands at 912.51 and 759.59 cm^{-1} were

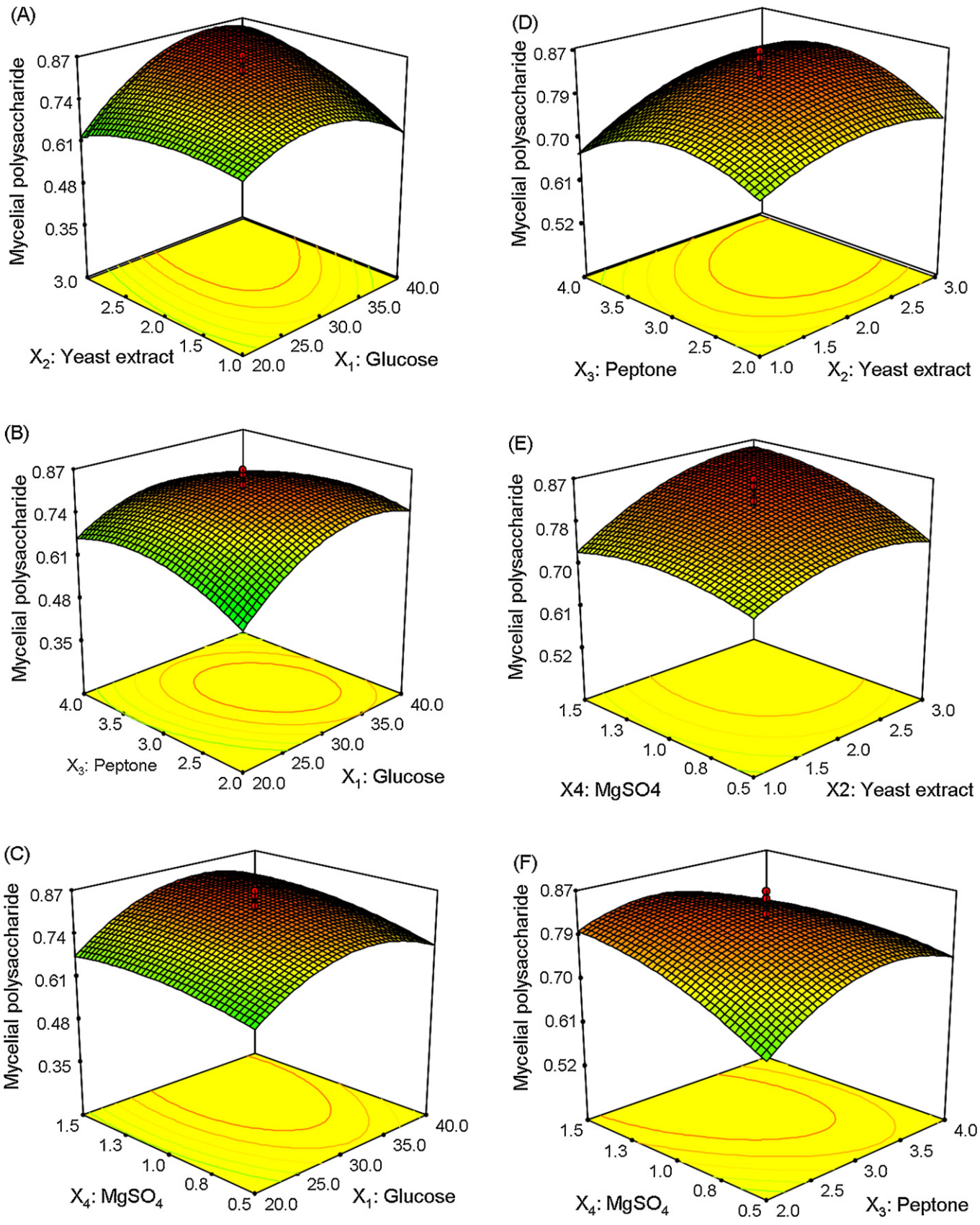

Fig. 1. 3D-response surface plots showing the interactive effects of varied glucose and yeast extract concentrations at 3 g/l peptone and 1 g/l $MgSO_4$ (A), varied glucose and peptone concentrations at 2 g/l yeast extract and 1 g/l $MgSO_4$ (B), varied glucose and $MgSO_4$ at 2 g/l yeast extract and 3 g/l peptone (C), varied peptone and yeast extract at 30 g/l glucose and 1 g/l $MgSO_4$ (D), varied yeast extract and $MgSO_4$ at 30 g/l glucose and 3 g/l peptone (E), and varied peptone and $MgSO_4$ at 30 g/l glucose and 2 g/l yeast extract (F) on the production of PBMP.

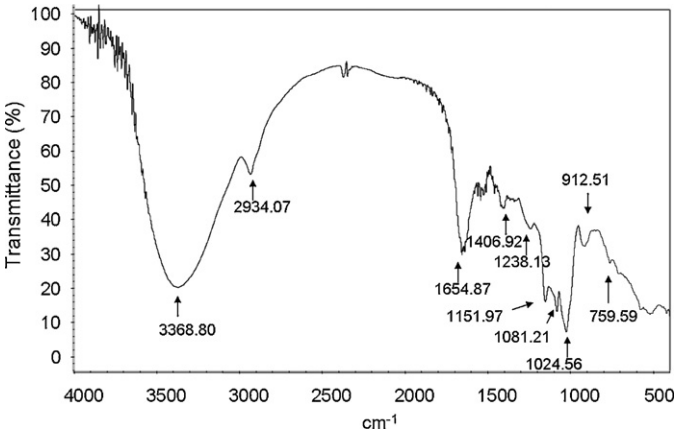

Fig. 2. FT-IR spectrum of the mycelial polysaccharide from *Phellinus baumii* Pilát in submerged culture.

characteristic signals for D-pyranose (Luo, Xu, Yu, Yang, & Zheng, 2008).

3.4. Antioxidant activity of PBMP in vivo

It has been reported that exopolysaccharides from submerged mycelial culture of *P. baumii* Pilát showed anti-diabetic effect or immuno-stimulating activity (Hwang et al., 2005; Luo et al., 2009). However, little information about the antioxidant activities of mycelial polysaccharides from submerged culture of *P. baumii* Pilát is available. Therefore, we investigated the antioxidant activity of PBMP *in vivo* by using the D-Gal induced aging mice model.

Recently, D-Gal has been widely used to induce oxidative stress *in vivo* to mimic natural aging in mice for screening antioxidant, anti-aging and neuroprotective drugs (Ho, Liu, & Wu, 2003; Hsieh, Wu, & Hu, 2009; Qiao et al., 2009; Sun, Yu, Zhang, Wang, & Luo, 2007). The long-term administration of D-Gal in mice can induce redox-related changes, cause accumulation of reactive oxygen species (ROS), or stimulate free radical production indirectly by the formation of advanced glycation end-product and further amplify oxidative stress (Xu & Zhao, 2002; Zhang, Wang, Zuo, & Cui, 2004). In this study, therefore, D-Gal-treated mice were used as an aging animal model for antioxidant evaluation *in vivo*. As shown in Table 5, the activities of antioxidant enzymes (SOD, GSH-Px, CAT) and the level of TAOC in serums of the D-Gal-treated mice were significantly decreased compared with those of the normal control group (Group I) ($p < 0.05$). However, MDA significantly increased compared to that of the normal control ($p < 0.05$). Thus, the results suggested that the aging mice model in the present study was established successfully.

The effects of PBMP on the activities of SOD, CAT, GSH-Px, TAOC, and the level of MDA in serums and livers of D-Gal-induced aging mice were determined (Tables 5 and 6). Compared with the D-Gal model control, the treatments by PBMP (especially at a dose of 200 or 400 mg/kg BW) significantly ($p < 0.01$) enhanced the activities of SOD, CAT, GSH-Px, and the level of TAOC and decreased markedly ($p < 0.01$) the concentration of MDA in a dose-dependent manner. Moreover, PBMP at a dose of 400 mg/kg BW showed better effect than Vc at a dose of 100 mg/kg BW when compared with the D-Gal model control. Therefore, the results demonstrated that PBMP could increase the activities of antioxidant enzymes and TAOC and decrease the level of MDA in livers and serums of mice to some extent, indicating that it has antioxidant activity *in vivo*.

Many studies have illustrated that aging is associated with the decrease in antioxidant status, and that age-dependent increments in lipid peroxidation are a consequence of diminished antioxidant protection (Hagihara, Nishigaki, Maseki, & Yagi, 1984; Schuessel et al., 2006). Crucial components of the antioxidant defense system in the body are cellular antioxidant enzymes (SOD, GSH-Px and CAT), which are involved in the reduction of ROS and peroxides produced in the living organism as well as in the detoxification of certain compounds of exogenous origin, thus playing a primary role in the maintenance of a balanced redox status. SOD reduces superoxide radicals (O_2^-) into H_2O_2 plus O_2, thus participating with other antioxidant enzymes in the enzymatic defense against oxygen toxicity. GSH-Px and CAT catalyze the reduction of H_2O_2 to H_2O and O_2, thereby preventing the formation of hydroxyl radicals (Yao et al., 2005). Hence, these enzymes act cooperatively at different sites in the metabolic pathway of free radicals. The data obtained in the present study showed that the activities of antioxi-

Table 5
Effects of PBMP on the activities of SOD (U/ml), CAT (U/ml), GSH-Px (U/ml), TAOC (U/ml) and levels of MDA (nmol/ml) in serums in aging mice.

Groups	SOD	CAT	GSH-Px	TAOC	MDA
I	283.16 ± 29.65[b]	99.18 ± 15.69[b]	184.37 ± 29.25[b]	38.91 ± 10.70[a]	11.45 ± 1.07[a]
II	187.62 ± 31.87	55.86 ± 9.73	102.07 ± 11.47	21.52 ± 4.81	15.27 ± 1.99
III	244.51 ± 18.09[a]	80.36 ± 17.18[b]	195.12 ± 28.92[b]	33.29 ± 0.69[b]	12.27 ± 2.75
IV	249.46 ± 29.52[a]	70.49 ± 9.98	208.48 ± 8.44[b]	32.58 ± 1.60[b]	8.73 ± 2.60[a]
V	266.14 ± 23.55[b]	96.21 ± 14.10[b]	232.80 ± 15.92[b]	35.45 ± 1.51[b]	7.27 ± 1.92[b]
VI	298.45 ± 25.95[b]	120.26 ± 17.58[b]	278.19 ± 16.81[b]	36.80 ± 3.14[b]	6.82 ± 1.54[b]

I, normal control; II, D-Gal model control; III, vitamin C as positive control; IV, V and VI, PBMP groups at a dose of 100, 200 and 400 mg/kg BW, respectively.
[a] Significantly different from the model group, $p < 0.05$.
[b] Significantly different from the model group, $p < 0.01$.

Table 6
Effects of PBMP on the activities of SOD (U/mg protein), CAT (U/mg protein), GSH-Px (U/mg protein), TAOC (U/mg protein) and levels of MDA (nmol/mg protein) in livers in aging mice.

Groups	SOD	CAT	GSH-Px	TAOC	MDA
I	514.80 ± 10.10[b]	49.61 ± 12.17[a]	1926.97 ± 72.23[b]	1.20 ± 0.22[b]	12.10 ± 1.45[a]
II	469.76 ± 21.15	31.80 ± 6.42	1616.10 ± 72.60	0.52 ± 0.07	17.15 ± 2.43
III	492.48 ± 15.19	63.23 ± 8.82[b]	2024.97 ± 179.30[b]	1.14 ± 0.19[b]	9.19 ± 2.66[b]
IV	480.81 ± 18.67	41.63 ± 12.57	2023.08 ± 125.21[b]	0.92 ± 0.19[b]	11.57 ± 1.69[b]
V	514.32 ± 11.49[b]	58.61 ± 7.43[b]	2938.01 ± 208.66[b]	1.07 ± 0.10[b]	9.65 ± 1.26[b]
VI	542.06 ± 16.69[b]	68.48 ± 11.07[b]	3294.34 ± 213.47[b]	1.30 ± 0.24[b]	7.82 ± 1.27[b]

I, normal control; II, D-Gal model control; III, vitamin C as positive control; IV, V and VI, PBMP groups at a dose of 100, 200 and 400 mg/kg BW, respectively.
[a] Significantly different from the model group, $p < 0.05$.
[b] Significantly different from the model group, $p < 0.01$.

dant enzymes, including SOD, CAT and GSH-Px, in serums and livers were dramatically decreased with aging by the treatment of D-Gal. Interestingly, treatments with PBMP could improve markedly the activities of those antioxidant enzymes in serums and livers of D-Gal-treated mice. These results suggested that the decrease in the activities of those antioxidant enzymes might be the main factor in lipid peroxidative damage. Meanwhile, our results suggested that PBMP supplementation effectively attenuated the oxidative damage and improved parameters related to aging.

The level of MDA, which indicates the degree of endogenous lipid peroxidation, is an oxidative stress marker (Freeman & Crapo, 1981). Lower MDA level suggests that there is less lipid peroxidation and weaker oxidant stress (Bagchi, Bagchi, Hassoun, & Stohs, 1995). Through antioxidant assay *in vivo*, we found that PBMP could inhibit markedly the increase of MDA content in serums and livers of D-Gal induced mice. In addition, the aging-related decrease in TAOC level suggests that the decrease in the nonenzymatic antioxidant defense probably also contributes to endogenous lipid peroxidation.

4. Conclusions

In the present study, FFD and CCD were employed to optimize the medium for PBMP production. As a result, an optimized medium was obtained as follows (g/l): glucose 35.36, yeast extract 2.88, peptone 2.73, $MgSO_4$ 1.47, KH_2PO_4 1, VB_1 0.0075 and diammonium oxalate monohydrate 0.3. Under the optimized conditions, the yield of PBMP reached 0.927 g/l. Through GC analysis, we found that the resulting PBMP was composed of D-glucose, D-galactose, L-fucose, D-mannose, and L-rhamnose in a molar ratio of 12.74:1.39:1.00:1.92:0.22. In addition, the assay of the antioxidant activity *in vivo* demonstrated that PBMP administration obviously increased the activities of antioxidant enzymes, enhanced total antioxidant capacity, and reduced the level of MDA in serums and livers of D-Gal-induced aging mice. These results suggested that PBMP had potent antioxidant activity and could be explored as novel natural antioxidant.

Acknowledgement

This work was partly supported by a grant-in-aid for scientific research from the National Natural Science Foundation of China (No. 30870547).

References

Bagchi, D., Bagchi, M., Hassoun, E. A., & Stohs, S. J. (1995). *In vitro* and *in vivo* generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides. *Toxicology*, *104*, 129–140.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New Method for quantitative determination of uronic acids. *Analytical Biochemistry*, *54*, 484–489.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254.

Chang, Z. Q., Oh, B. C., Rhee, M. H., Kim, J. C., Lee, S. P., & Park, S. C. (2007). Polysaccharides isolated from *Phellinus baumii* stimulate murine splenocyte proliferation and inhibit the lipopolysaccharide-induced nitric oxide production in RAW264.7 murine macrophages. *World Journal of Microbiology and Biotechnology*, *23*, 723–727.

Chen, W., Zhao, Z., Chen, S. F., & Li, Y. Q. (2008). Optimization for the production of exopolysaccharide from *Fomes fomentarius* in submerged culture and its antitumor effect *in vitro*. *Bioresource Technology*, *99*, 3187–3194.

Dong, C. H., & Yao, Y. J. (2005). Nutritional requirements of mycelial growth of *Cordyceps sinensis* in submerged culture. *Journal of Applied Microbiology*, *99*, 483–492.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350–356.

Freeman, B. A., & Crapo, J. D. (1981). Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. *Journal of Biological Chemistry*, *256*(21), 10986–10992.

Ge, Q., Zhang, A., & Sun, P. (2009). Structural investigation of a novel water-soluble heteropolysaccharide from the fruting bodies of *Phellinus baumii* Pilát. *Food Chemistry*, *114*, 391–395.

Guerrant, G. O., & Moss, C. W. (1984). Determination of monosaccharides as aldononitrile, *O*-methyloxime, alditol, and cyclitol acetates derivatives by gas chromatography. *Analytical Chemistry*, *56*, 633–638.

Hagihara, M., Nishigaki, I., Maseki, M., & Yagi, K. (1984). Age-dependent changes in lipid peroxide levels in the lipoprotein fractions of human serum. *Journal of Gerontology*, *39*, 269–272.

Ho, S. C., Liu, J. H., & Wu, R. Y. (2003). Establishment of the mimetic aging effect in mice caused by D-galactose. *Biogerontology*, *4*, 15–18.

Hsieh, H. M., Wu, W. M., & Hu, M. L. (2009). Soy isoflavones attenuate oxidative stress and improve parameters related to aging and Alzheimer's disease in C57BL/6J mice treated with D-galactose. *Food and Chemical Toxicology*, *47*(3), 625–632.

Hwang, H. J., Kim, S. W., Lim, J. M., Joo, J. H., Kim, H. O., Kim, H. M., et al. (2005). Hypoglycemic effect of crude exopolysaccharides produced by a medicinal mushroom *Phellinus baumii* in streptozotocin-induced diabetic rats. *Life Sciences*, *76*, 3069–3080.

Jang, B. S., Kim, J. C., Bae, J. S., Rhee, M. H., Jang, K. H., Song, J. C., et al. (2004). Extracts of *Phellinus gilvus* and *Phellinus baumii* inhibit pulmonary inflammation induced by lipopolysaccharide in rats. *Biotechnology Letters*, *26*, 31–33.

Ke, C., Qiao, D., Gan, D., Ye, H., Sun, Y., Yan, X., et al. (2009). Antioxidant acitivity *in vitro* and *in vivo* of the capsule polysaccharides from *Streptococcus equi* subsp. Zooepidemicus. *Carbohydrate Polymers*, *75*(4), 677–682.

Kim, S. W., Hwang, H. J., Xu, C. P., Na, Y. S., Song, S. K., & Yun, J. W. (2002). Influence of nutritional conditions on the mycelial growth and exopolysaccharide production in *Paecilomyces sinclairii*. *Letter in Applied Microbiology*, *34*, 389–393.

Lee, B. C., Bae, J. T., Pyo, H. B., Choe, T. B., Kim, S. W., & Hwang, H. J. (2004). Submerged fermentation conditions for the production of mycelial biomass and exopolysaccharides by the edible Basidiomycete *Grifola frondosa*. *Enzyme and Microbial Technology*, *35*, 369–376.

Liu, J. C., Miao, S., Wen, X. C., & Sun, Y. X. (2009). Optimization of polysaccharides (ABP) extraction from the fruiting bodies of *Agaricus blazei* Murill using response surface methodology (RSM). *Carbohydrate Polymers*, *78*(4), 704–709.

Luo, J., Liu, J., Ke, C., Qiao, D., Ye, H., Sun, Y., et al. (2009). Optimization of medium composition for the production of exopolysaccharides from *Phellinus baumii* Pilát in submerged culture and the immuno-stimulating activity of exopolysaccharides. *Carbohydrate Polymers*, *78*(3), 409–415.

Luo, X., Xu, X. Y., Yu, M., Yang, Z. R., & Zheng, L. Y. (2008). Characterisation and immunostimulatory activity of an α-(1,6)-D-glucan from the cultured *Armillariella tabescens* mycelia. *Food Chemistry*, *111*, 357–363.

Mao, X. B., Eksriwong, T., Chauvatcharin, S., & Zhong, J. J. (2005). Optimization of carbon source and carbon/nitrogen ratio for cordycepin production by submerged cultivation of medicinal mushroom *Cordyceps militaris*. *Process Biochemistry*, *40*(5), 1667–1672.

Muralidhar, R. V., Chirumamila, R. R., Marchant, R., & Nigam, P. (2001). A response surface approach for the comparison of lipase production by *Candida cylindracea* using two different carbon sources. *Biochemical Engineering Journal*, *9*(1), 17–23.

Qiao, D., Ke, C., Hu, B., Luo, J., Ye, H., Sun, Y., et al. (2009). Antioxidant activities of polysaccharides from *Hyriopsis cumingii*. *Carbohydrate Polymers*, *78*(2), 199–204.

Schuessel, K., Frey, C., Jourdan, C., Keil, U., Weber, C. C., Müller-Spahn, F., et al. (2006). Aging sensitizes toward ROS formation and lipid peroxidation in PS1M146L transgenic mice. *Free Radical Biology and Medicine*, *40*(5), 850–862.

Shih, I. L., Pan, K., & Hsieh, C. (2006). Influence of nutritional components and oxygen supply on the mycelial growth and bioactive metabolites production in submerged culture of *Antrodia cinnamomea*. *Process Biochemistry*, *41*, 1129–1135.

Shon, M. Y., Kim, T. H., & Sung, N. J. (2003). Antioxidants and free radical scavenging activity of *Phellinus baumii* (Phellinus of Hymenochaetaceae) extracts. *Food Chemistry*, *82*, 593–597.

Sun, S. W., Yu, H. Q., Zhang, H., Wang, J., & Luo, L. (2007). Quercetin attenuates spontaneous behavior and spatial memory impairment in D-galactose-treated mice by increasing brain antioxidant capacity. *Nutrition Research*, *27*(3), 169–175.

Xu, X. H., & Zhao, T. Q. (2002). Effects of puerarin on D-galactose-induced memory deficit in mice. *Acta Pharmacologica Sinica*, *23*, 587–590.

Yao, D. C., Shi, W. B., Gou, Y. L., Zhou, X. R., Tak, Y. A., & Zhou, Y. K. (2005). Fatty acid mediated intracellular iron translocation: A synergistic mechanism of oxidative injury. *Free Radical Biology and Medicine*, *39*, 1385–1398.

Zhang, C., Wang, S. Z., Zuo, P. P., & Cui, X. (2004). Protective effect of tetramethylpyrazine on learning and memory function in D-galactose lesioned mice. *Chinese Medical Sciences Journal*, *19*(3), 180–184.

Zhao, M. M., Yang, N., Yang, B., Jiang, Y. M., & Zhang, G. H. (2007). Structural characterization of water-soluble polysaccharides from *Opuntia monacantha* cladodes in relation to their anti-glycated activities. *Food Chemistry*, *105*, 1480–1486.