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

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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.

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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.