Optimal Conditions of Mycelial Growth and Exopolysaccharide Production in Submerged Culture of *Phellinus baumii*

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The polysaccharide isolated from *Phellinus* species has been known as a folk remedy, including anti-tumor and immuno-stimulating activities. However, there are lacks of knowledge about mycelial growth and exopolysaccharide (EPS) production in its submerged culture. We investigated the optimal conditions on mycelial growth and EPS production in *Phellinus baumii*. The optimal temperature and initial pH for mycelial growth and EPS production in shake flask culture of *P. baumii* were proved to be 30°C and pH 5.0, respectively. In case of carbon source, cellobiose and maltose were highly efficient for mycelial growth and fructose and mannitol were also relatively favorable for EPS production. Yeast extract was the most suitable nitrogen source for mycelial growth and EPS production. The composition of optimal culture medium was determined to be fructose 20 g/L, yeast extract 20 g/L, and $CaCl_2$ 0.55 g/L, respectively. Under the optimal culture condition, the maximum mycelial biomass and EPS achieved in a 5-L stirred-tank fermenter were 17.43 g/L and 3.6 g/L, respectively. It was found that the EPS was a glycoprotein consisted of mainly arginine (14.1%) and glycine (12.0 %) in protein moiety and mainly mannose (48.7%) and arabinose (38.4%) in carbohydrate moiety.

Key words – exopolysaccharide, mycelial growth, *Phellinus baumii*, submerged culture

The practice using macrofungi in previous studies, especially mushrooms to treat a variety of disease, has been performed in many countries [9,26]. During the past decades, many researchers have reported that crude polysaccharides obtained from the mycelial culture of fruiting body stimulated various pharmacological and biological activities, including anti-tumor, immuno-stimulating, and hypoglycemic activities, etc. [16,21,23,31,34,35]. In order to obtain polysaccharides from mushrooms, most of investigators have exerted their efforts to cultivate edible and medicinal mushrooms on solid artificial media (for fruit body production) rather than submerged culture (for mycelial extract and/or EPS production) [2,3,20]. Submerged culture obviously gives rise to potential advantages of higher mycelial production in a compact space and shorter period without significant problem of contamination [1,6,28,33]. Although enormous efforts have been made to obtain optimal conditions of submerged culture for bioactive polysaccharide production from several mushrooms, the currently available reports for nutritional requirements and environmental conditions in submerged cultures are quite limited to a few kinds of mushrooms [19,27,32].

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At present, many groups of *Phellinus* (e.g. *P. igniarius, P. hartigii, P. gilvus, P. pini,* etc.) are known and they have a wide variety of medicinal effects such as anti-tumor and immuno-stimulating activities [14,18,29,30]. Amongst them, *P. linteus* has been well known as a medicinally potent mushroom due to its strong anti-tumor activity [7,10,12].

In the course of investigation of several *Phellinus* species, it was found that *P. baumii* had a capability of producing a considerable amount of EPS. *P. baumii* is a mushroom used as folk medicine for a variety of human diseases, such as diabetes and cancer in Korea. Although there are many reports on polysaccharides extracted from mycelia of *Phellinus* species, any useful data about extracellular polysaccharides obtained from culture filtrates of *Phellinus* species are scarcely available. To date, any reports about submerged cultures of *P. baumii* are not currently available.

In this study, in order to investigate factors affecting on the production of mycelial biomass and EPS, submerged culture of *P. baumii* was carried out and various culture conditions were investigated.

Materials and Methods

Microorganism

P. baumii was kindly provided from Non-Gong Mushroom

Co. (Kyungbuk, Korea). The stock culture was inoculated on potato dextrose agar (PDA) slant, incubated at 28℃ for 6 days, and used for experiments. The stock culture was maintained by subculturing every month and the slants were stored at 4℃.

Inoculum preparation

P. baumii was initially grown on PDA medium in a petri dish and then transferred to the seed culture medium by punching out a portion (5 mm diameter) of the agar plate with a sterilized self-designed cutter. The seed culture was grown in a 250 ml flask containing 50 ml of PMP medium (2.4% potato dextrose broth, 1% malt extract, 0.1% peptone) at 28℃ with shaking at 150 rpm for 4 days.

Culture conditions

The flask culture experiments were performed in a 250 ml flask containing 50 ml of the medium for 8 days, after inoculating with 5% (v/v) of the seed culture. The fermentation experiments were also carried out in a 5 L stirred-tank fermenter (KoBioTech Co., Seoul, Korea) containing 3 L of optimal medium. The culture was inoculated with 5% (v/v) of the seed culture and incubated under the following conditions: temperature, 30℃; aeration rate, 2 vvm; agitation speed, 150 rpm; initial pH 5.0; working volume, 3 L. All experiments were carried out in triplicate and the data were expressed as mean±SD of triple determinations.

Estimation of mycelial and polysaccharide concentrations

Samples collected from shake flasks at various intervals were centrifuged at 10,000×*g* for 20 min and the resulting supernatant was filtered through a Whatman filter paper No. 2 (Whatman International Ltd., Maidstone, England). The filtrate was mixed with 4 times volume of absolute ethanol, stirred vigorously and stand overnight at 4℃. The precipitated EPS was centrifuged at 10,000×*g* for 20 min and the supernatant discarded. The precipitate of pure EPS was lyophilized and the weight was estimated. Dry weight of the mycelium was measured after repeated washing of the mycelial pellet with distilled water and drying at 90℃ overnight to a constant weight (Fig. 1). For quantitative assay of residual sugars, the filtrate through a membrane filter was analyzed by HPLC (Shimadzu Co., Osaka, Japan) using an Aminex HPX42C column equipped with a refractive index detector.

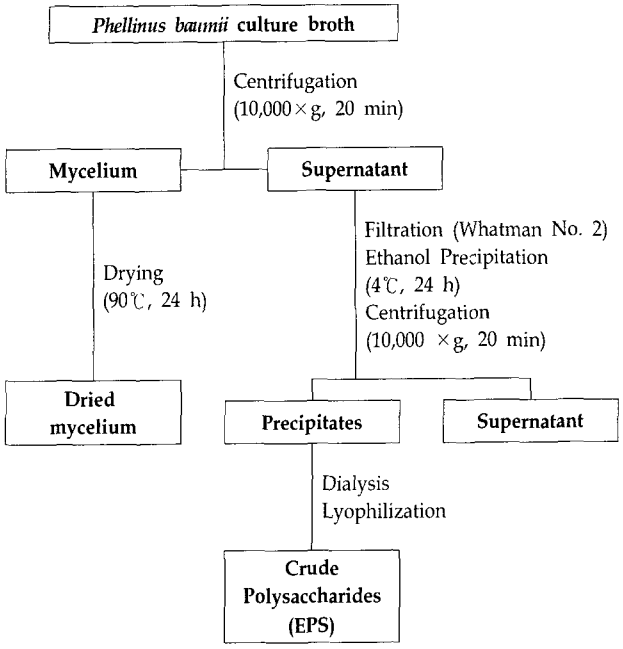

Fig. 1. Recovery process of the polysaccharides produced by submerged culture of *P. baumii*.

Analysis of carbohydrates and amino acids

Total sugar content of EPS produced from *P. baumii* was determined by phenol sulfuric acid method using glucose as the standard [8]. Sugar composition was analyzed by gas chromatography (Varian Co., Model: Star 3600CX, Lexington, MA, USA) with a fused silica capillary column (Na form, 300×0.25 mm, Supelco Inc., Bellefonte, PA, USA) and a flame ionization detector. Total protein was determined by the Lowry method with bovine serum albumin as the standard [24]. The composition of amino acid was analyzed by amino acid analyzer (Biochrom Ltd., Model: Biochrom 20, Cambridge, UK) with high performance ion exchange column (No. 3906, 200×4.6 mm).

Results and Discussion

Effects of temperature and initial pH

To investigate optimal temperature for mycelial growth and EPS production, *P. baumii* was cultivated in shake flask at various temperatures (20-35℃) using PMP medium, where the optimum temperature was determined to be 30℃ (Fig. 2A). The optimal temperature is quite similar to the results reported by Chi et al. [4] and Kang et al. [15] from other liquid cultures of different species of *Phellinus*. It is a contrast to other data, in which many kinds of mushrooms have relatively low temperature optima ranging from 20 to

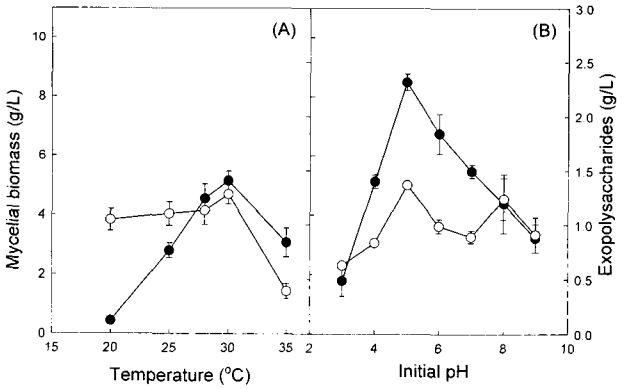

Fig. 2. Effects of temperature (A) and initial pH (B) on the mycelial biomass and exopolysaccharide production by submerged culture of *P. baumii*. (●) mycelial biomass, (○) exopolysaccharides. Experiments were carried out using PMP medium. All experimental data were mean±SD of triple determinations.

25℃ in their submerged cultures [2,27].

Generally, the acidity of culture broth is one of the most critical environmental parameters affecting mycelial growth and biosynthesis of EPS in submerged cultures. In order to investigate the effect of initial pH on mycelial growth and EPS production, *P. baumii* was cultivated under different initial pHs (3.0-9.0) in shake flask. The highest levels of mycelial biomass and EPS production were obtained at pH 5 (Fig. 2B).

It has been reported that a variety of mushrooms have also acidic pH optima [19,27] and particularly *P. linteus* has pH optima at pH 5-7 [5,17]. Lee et al. [22] found an important result that the culture pH affects the monosaccharide composition in polysaccharide; i.e., the amount of glucose was decreased from 90% to 83% by changing culture pH from 5.0 to 9.0.

Effects of carbon and nitrogen source

To find a suitable carbon source for the EPS production in *P. baumii*, mycelia were cultivated in the medium containing various carbon sources at a final concentration of 1% (w/v) for 8 days. Among 9 carbon sources examined, cellobiose and maltose were highly efficient for mycelial growth, while fructose and mannitol were also relatively favorable for EPS production (Fig. 3). Although cellobiose and maltose showed good mycelial growth, they led to low EPS yields. These results indicated that the nutritional requirement for EPS production in *P. baumii* was not always consistent with that of mycelial growth. In this study, fructose was selected as a suitable carbon source and its optimum

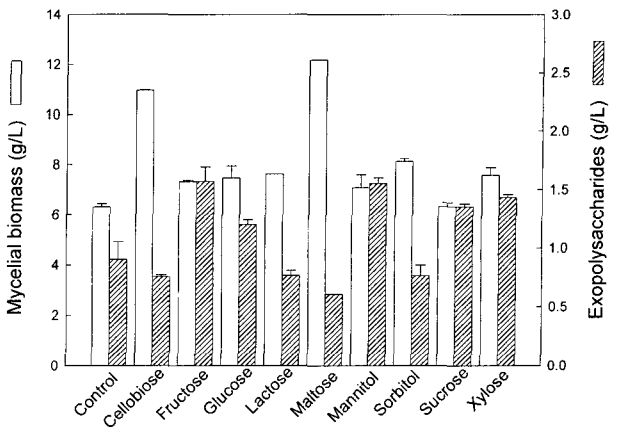

Fig. 3. Effect of carbon source on the mycelial biomass and exopolysaccharide production by submerged culture of *P. baumii*. Control means PMP medium without addition of potato dextrose broth.

concentration was examined to be 20 g/L (data not shown).

Yeast extract from 11 kinds of nitrogen sources tested was chosen as the most effective nitrogen source for both mycelial growth and EPS production. The optimum concentration of yeast extract was determined to be 20 g/L (data not shown). Chi et al. [4] pointed out that several amino acids were more favorable nitrogen sources than complex nitrogen sources for mycelial growth of *P. linteus*. In comparison with organic nitrogen sources, inorganic nitrogen sources gave rise to relatively lower mycelial biomass and EPS production. It has been frequently suggested that certain essential amino acids could scarcely be synthesized from inorganic nitrogen sources during the fermentation process of higher fungi. Hence, it is probable that most *basidomycetes* require complex organic nitrogen source for their favorable submerged cultures [7,33].

Effect of bioelement

In several fungal fermentations, mineral ions are usually recognized as one of efficient nutrients for mycelial growth and production of fungal metabolites [27]. In this study, supplementation of calcium ions led to increase the mycelial growth and EPS production, but other mineral ions had negligible effects except ferrous and manganese ions (Table 1). Jonathan and Fasidi [13] suggested that macroelements (Mg, K, and Ca) promoted mycelial growth of *Lentinus subnudus* (Berk) and *Schizophyllum commune* and Kang et al. [15] have reported that KH_2PO_4 and $CaCl_2$ were the most effective mineral sources for mycelial growth of *Phellinus* sp. Accordingly, calcium ion might be requisite for mycelial growth and EPS production of *P. baumii*. The optimal con-

Table 1. Effect of mineral sources on the mycelial biomass and exopolysaccharide production of *P. baumii*[*,†]

Mineral sources (0.005 M)	Mycelial biomass (g/L)	Exopolysaccharides (g/L)	Final pH
Control[‡]	5.14±0.15	1.64±0.03	5.12
$CaCl_2$	8.16±0.22	2.26±0.12	5.29
$FeSO_4$	1.74±0.21	1.11±0.17	4.61
KH_2PO_4	6.51±0.31	1.65±0.23	5.13
K_2HPO_4	6.18±0.34	1.83±0.20	5.19
$MgSO_4$	5.12±0.62	2.12±0.30	4.92
$MnSO_4$	1.07±0.11	0.98±0.08	5.15

*The flask culture experiments were carried out for 8 d.
†Values are mean±SD of triple determinations.
‡Control means the medium containing 2% fructose, 2% yeast extract without addition of mineral source.

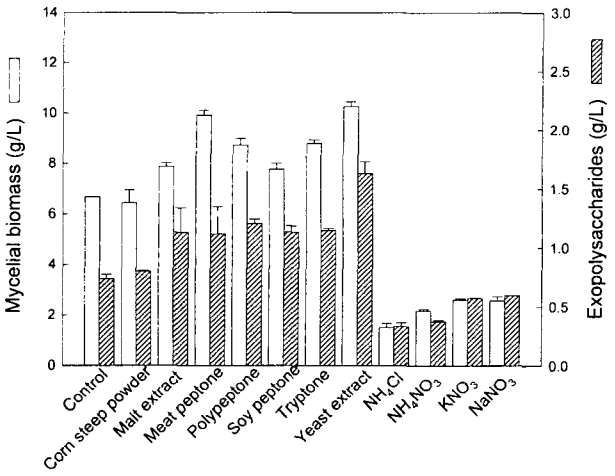

Fig. 4. Effect of nitrogen source on the mycelial biomass and exopolysaccharide production by submerged culture of *P. baumii*. Control means PMP medium without addition of malt extract and peptone.

centration of $CaCl_2$ was proved to be 0.55 g/L (data not shown).

Fermentation results

Fig. 5 shows the typical time profiles of substrate consumption, mycelial growth, and EPS production in a 5-L stirred-tank fermenter under the optimized culture conditions. The mycelial growth was continuously increased during fermentation process and the maximum concentration of mycelium reached to 17.4 g/L at day 14, whereas the maximum EPS production (3.6 g/L) was achieved at day 16. The initial acidity (pH 5.0) of the fermentation broth was kept nearly constant during the first 12 days and then slowly increased to pH 7.3 at the end of fermentation. This

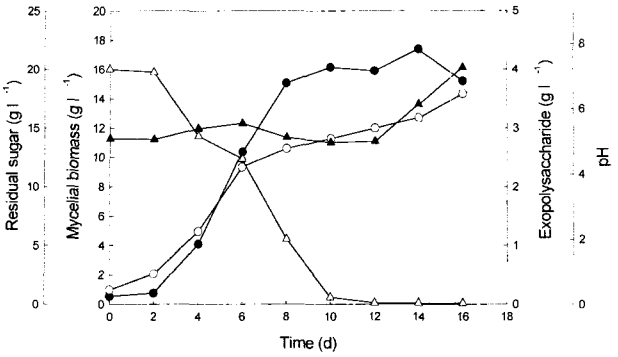

Fig. 5. Typical time profiles for the mycelial biomass and exopolysaccharide production by submerged culture of *P. baumii* in a 5-L stirred-tank fermenter. (●) mycelial biomass, (○) exopolysaccharides, (▲) pH, (△) residual sugar.

increment in the later stage of fermentation is an unusual phenomenon, compared to those occurred in most liquid-cultures of mushrooms [11,27]. It is thought that the pH change might be caused by fragmentation of the mycelia at the later stage of fermentation.

Compositional analysis of the crude EPS

The detailed compositions of carbohydrate and amino acid in crude EPS were illustrated in Table 2. The EPS was proved to be a glycoprotein consisting of mainly arginine (14.1%) and glycine (12.0%) in protein moiety and mainly mannose (48.7%) and arabinose (38.4%) in carbohydrate

Table 2. Amino acid and carbohydrate composition in exopolysaccharides produced from submerged culture of *P. baumii*

Amino acid	Composition (%)	Carbohydrate	Composition (%)
Aspartic acid	11.36	Maltose	4.1
Threonine	6.72	Arabinose	38.43
Serine	7.29	Xylose	2.86
Glutamic acid	10.80	Mannose	48.69
Glycine	12.03	Galactose	2.64
Alanine	5.21	Glucose	3.29
Cystine	2.46		
Valine	5.59		
Methionine	1.23		
Isoleucine	2.94		
Leucine	3.69		
Tyrosine	2.75		
Phenylalanine	6.06		
Histidine	1.89		
Lysine	5.87		
Arginine	14.11		

moiety. The amino acid moiety of the glycoprotein might be related to the anti-tumor and immuno-stimulating activities [25]. Using the purified EPS, in order to elucidate, which moiety of the glycoprotein has pharmacological or biological activity, further study will be performed.

Acknowledgment

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초록 : *Phellinus baumii*으로부터 세포외 다당체 생산의 최적화

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장수상황버섯(*Phellinus baumii*)의 균사체 성장 및 세포외 다당체(exopolysaccharides) 생산을 위한 액체배양의 최적 배지 및 배양조건에 관한 실험을 수행한 결과, 최적배양 온도와 초기 pH는 각각 30℃와 5.0으로 결정되었다. 탄소원의 경우, cellobiose 와 maltose 가 균사체 성장에는 양호하였으나 저조한 세포외 다당체 생산을 보였으며, 반면에 fructose 와 mannitol 은 상대적으로 높은 수율의 세포외 다당체 생산과 균사체 성장 또한 양호하였다. 최적 질소원으로는 yeast extract 가 균사체 성장뿐만 아니라 세포외 다당체 생산에 효과적인 것으로 결정되었다. 최적 배지의 조성은 fructose 20 g/L, yeast extract 20 g/L 및 $CaCl_2$ 0.55 g/L이었으며, 최적 배양조건 하에서 5-L 교반 발효조를 운전한 결과, 최대 균사체 성장(17.43 g/L)과 최대 세포외 다당체 생산(3.6 g/L)을 얻을 수 있었다. 회수된 세포외 다당체는 glycoprotein이었으며 그 조성은 amino acid 분석 결과 주로 arginine (14.1%)과 glycin (12.0%)이었으며, cabohydrate의 경우는 mannose (48.7%)와 arabinose (38.4%)이 주성분이었다.