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Culture pH Affects Exopolysaccharide Production in Submerged Mycelial Culture of *Ganoderma lucidum*

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Abstract

In submerged culture of *Ganoderma lucidum*, the pH optimum for cell growth has been shown to be lower than that for exopolysaccharides (EPS) formation. Therefore, in the present study, a two-stage pH-control strategy was employed to maximize the productions of mycelial biomass and EPS. When compared, a batch culture without pH control had a maximum concentration of EPS and endopolysaccharides, which was much lower than those with pH control. Maximum mycelial growth (12.5 g/L) and EPS production (4.7 g/L) were achieved by shifting the controlled pH from 3.0 to 6.0 after day 4. The contrast between the controlled-pH process and uncontrolled pH was marked. By using various two-stage culture processes, it was also observed that culture pH has a significant affect on the yield of product, mycelial morphology, chemical composition, and molecular weight of EPS. A detailed observation of mycelial morphology revealed that the productive morphological form for EPS production was a dispersed pellet (controlled pH shifting from 3.0 to 6.0) rather than a compact pellet with a dense core area (controlled pH 4.5) or a feather-like pellet (controlled pH shifting from 6.0 to 3.0). Three different polysaccharides were obtained from each pH conditions, and their molecular weights and chemical compositions were significantly different.

Index Entries: Culture pH; exopolysaccharides; *Ganoderma lucidum*; mushrooms; submerged culture.

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Introduction

Ganoderma lucidum is one of the most well-known traditional medicinal mushrooms and has been widely used in Asian countries for years. The extracts from this mushroom have been intensively studied because of their wide range of biological activities, such as anti-cancer *(1,2)*, anti-allergy *(3)*, anti-inflammatory *(4–7)*, antioxidant *(8–10)*, blood pressure reducing *(11–13)*, hypoglycemic effects *(14,15*), and platelet-aggregation-inhibiting activities *(16,17)*. Recently, researchers have focused their attention on various traditional polysaccharide sources, as a result of the numerous side effects and toxicity raised by current synthetic drugs *(18,19)*.

Many investigators have tried to cultivate mushrooms on solid artificial media for fruit body formation in order to obtain bioactive polysaccharides *(20,21)*. However, this method does not guarantee a standardized product because its composition varies from batch to batch. As a result, recent attention has been paid to the use of submerged culture for the production of mycelial biomass and bioactive products, particularly extracellular polysaccharides (EPS) *(20,22,23)*.

There are many reports mentioning the various optimum submerged culture conditions of *G. lucidum* and their production of mycelial biomass and EPS *(24–31)*. Of the various culture conditions, culture pH is one of the most important environmental factors in fungal fermentation, because it can profoundly affect cell growth and metabolite production, as well as cell morphology and broth rheology *(26,31,32)*. There are several published data describing the influence of culture pH on the final concentration of bioactive products for submerged mushroom cultures *(24–26,33–35)*. However, most researchers focused mainly on the apparent fermentation results, such as product concentration and morphological changes, and did not pay special attention to the alterations in the quality of target products produced when the culture pH varied. The molecular weight, chemical composition, branching mode, and conformation of the mushroom polysaccharides have significant effects on its various biological activities *(36,37)*. In this regard, detailed molecular characterizations are essentially required for elucidating the correlation between physiochemical properties and bioactivities of polysaccharides.

In submerged cultures of *G. lucidum*, there are conflicting results regarding optimum pH for mycelial growth and EPS formation. However, in general, the pH for optimum cell growth has been shown to be lower than that for EPS formation *(24–26,30)*. This suggests the possibility of a two-stage fermentation process in which the pH is initially suited to hasten the period of biomass formation, followed by a phase at different pH, which is tailored to optimum EPS synthesis. Such a strategy has been successfully demonstrated for EPS production in *G. lucidum* and pullulan production *(26,35,38)*.

In the present study, a two-stage pH-control strategy was used to maximize the productions of mycelial biomass and EPS, and especially to

investigate how culture pH affects EPS quality, such as chemical compositions and molecular weight distribution.

Materials and Methods

Microorganism and Media

A culture of *G. lucidum* DG-6556, an isolate from our laboratory, was maintained on potato dextrose agar (PDA) slants and stored in a 25% glycerol solution at –70°C for about 2 mo. The slants were incubated at 25°C for 4 d, then stored at 4°C, and subcultured every 4 wk. The seed cultures were grown in 250-mL flasks containing 50 mL of mushroom complete medium ([MCM]; 20 g/L glucose, 2 g/L meat peptone, 2 g/L yeast extract, 0.46 g/L KH_2PO_4, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$) at 25°C on a rotary shaker incubator at 150 rpm for 4 d.

Inoculum Preparation and Culture Conditions

G. lucidum was initially grown on PDA medium in a Petri dish, and then transferred into the seed culture medium by punching out 5 mm of the agar plate culture with a house-developed cutter. The precultures were carried out in 250-mL flasks containing 50 mL of MCM at 25°C for 4 d, using 4% (v/v) inocula. All experiments were performed at least in triplicate to ensure reproducibility. Fermentations were carried out using a 5-L stirred-tank bioreactor (Ko-BioTech Co., Seoul, Korea) with a six-bladed turbine impeller and a working volume of 3 L. The aeration rate and rotation speed were 2 vvm and 150 rpm, respectively. The culture medium was the same as in the flask culture. Samples were taken every 2 d for analyses of mycelial dry weight, EPS and residual sugar concentration.

Estimation of Mycelial Growth, EPS, and ENPS Concentration

Samples were collected at various intervals from shake flasks and centrifuged at 10,000*g* for 20 min. The resulting supernatant was then filtered through a Whatman filter paper no. 2 (Whatman International Ltd., Maidstone, England). The resulting culture filtrate was mixed with four volumes of absolute ethanol, stirred vigorously and left overnight at 4°C. The EPS fractions formed on the surface of the solution (hereafter named "Top-EPS") was recovered using a stainless steel mesh (pore size: 45 µm) and the precipitated EPS (hereafter named "Bottom-EPS") was collected by centrifugation at 10,000*g* for 10 min. Both EPS were dialyzed overnight against distilled water, followed by lyophilization and weighed. After repeated washing of the mycelium with distilled water and drying at 70°C for 28 h to a constant weight, the dry weight of the mycelium was measured. For the analysis of endopolysaccharides (ENPS) concentration, the dried mycelia (100 mg) were extracted using 15 mL of 1 *N* NaOH at 65°C (for 3 h), followed by filtration (0.22 µm). The concentrations of ENPS and residual sugars were analyzed by the phenol-sulfuric acid method using glucose as the standard *(39)*.

Measurement of Morphology

The morphological details of samples were evaluated using an image analyzer (Matrox Electronic System Ltd., Dorval, Quebec, Canada) with software coupled to a light microscope (Olympus Optical Co., Ltd., Tokyo, Japan) and a charge-coupled device (CCD) camera (Matsushita Communication Industrial Co., Ltd., Yokohama, Japan). The CCD camera captured images of 512 × 512 pixels, each with grayness levels from 0 (black) to 255 (white). Samples were fixed with equal volumes of fixative (13 mL of 40% formaldehyde, 5 mL glacial acetic acid with 200 mL of 50% ethanol). Then 0.1 mL of each fixed sample was transferred to a slide, air dried, then stained with methylene blue (0.3 g of methylene blue and 30 mL of 95% ethanol in 100 mL water) *(40)*.

Compositional Analysis

The total sugar content of EPS and ENPS was determined by the phenol sulfuric acid method *(39)* using glucose as the standard. Monosaccharide components and their ratios were determined by hydrolysis of the polysaccharides with 2 *M* trifluroacetic acid at 121°C for 3 h. The hydrolysates were evaporated to dryness, followed by successive reduction with $NaBH_4$ and acetylation with Ac_2-O (1:1, v/v; 2 mL) at room temperature for 12 h *(41,42)*. Sugar composition was subjected to preparation of corresponding alditol acetate and then analyzed by gas chromatography (Varian Co., Model: Star 3600CX, Lexington, MA) using a fused silica capillary column (Na form, 300 ¥ 0.25 mm, Supelco Inc., Bellefonte, PA) and a flame ionization detector.

SEC/MALLS Analysis

The molecular weights of the EPS were estimated by size exclusion chromatography (SEC) coupled with a multiangle laser light-scattering (MALLS) system (DAWN DSP; Wyatt Technology, Santa Barbara, CA). The EPS samples were dissolved in a phosphate buffer (ionic strength = 0.1, pH 6.1) containing 0.04% ethylenediamine-tetraacetic acid-disodium salt (Na_2 EDTA) and 0.01% sodium azide and filtered through 0.025 μm filter membranes (Millex HV type; Millipore Co., Bedford, MA) prior to injection into the SEC/MALLS system *(43,44)*. The SEC system consisted of a degassor (Degasys, DG-1200, uniflow; HPLC Technology, Maccles-field, UK), a high-performance pump (Model 590 Programmable Solvent Delivery Module; Waters Co., Milford, MA), an injection valve (Rheodyne Inc., Cotati, CA) fitted with a 100-μL loop, the SEC columns (Shodex PROTEIN KW-803, 804; Showa Denko K.K., Tokyo, Japan) connected in series, and an RI detector (Waters 410).

Chromatography was performed at room temperature, where the flow rate, injection volume, and injection concentration were 0.5 mL/min, 100 μL, and 3 mg/mL, respectively. During calculation of the molecular weights of each EPS, the value of *dn/dc* (specific refractive index increment)

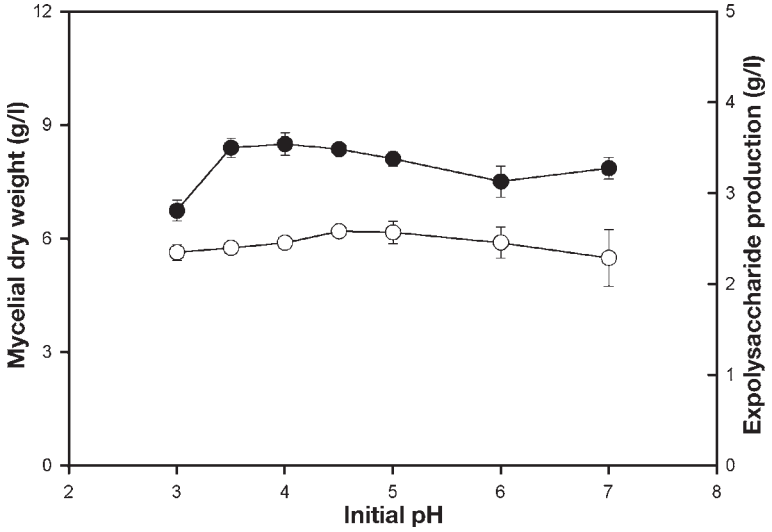

Fig. 1. Effect of initial pH on mycelial growth (●) and exopolysaccharide (○) production in submerged culture of *Ganoderma lucidum*.

was used according to the Wyatt Technology guide and literature data *(45)*. Calculation of molecular weights was performed using Astra 4.72 software (Wyatt Technology).

Results and Discussion

Effect of Initial pH on Mycelial Growth and EPS Formation

In order to investigate the effect of initial pH on mycelial growth and EPS formation, triplicate batch experiments were performed in flasks by varying initial culture pH from 3.0 to 7.0. As shown in Fig. 1, the optimum pH for cell growth was pH 4.0, whereas pH 5.0 was optimum for EPS formation. Considering the uncontrolled pH nature in flask cultures, these results indicate that mycelial growth might favor a lower culture pH range, and EPS formation a higher culture pH range. Thus, further experiments were carried out in stirred-tank fermenter at an initial pH 4.5, where the profiles of ENPS were also indicated for a comparative study (Fig. 2).

The maximum mycelial biomass (10.2 g/L) was achieved at day 10, whereas EPS and ENPS concentrations reached their maximum levels of 3.0 g/L at day 12 and 1.6 g/L at day 10, respectively. The maximum yield of EPS (about 0.5 g/g cell) and the maximum ratio of EPS to total polysaccharides (TPS) (about 78%) were observed in the middle of the exponential growth phase (from days 4 to 6). In contrast, the yield of ENPS (about 0.2 g/g cell) was not significantly altered throughout the entire fermentation period (Fig. 2B). The yields of EPS and ENPS, against consumed glucose, were determined to be 0.051 and 0.029 g/g/d when estimated during the first 6 d.

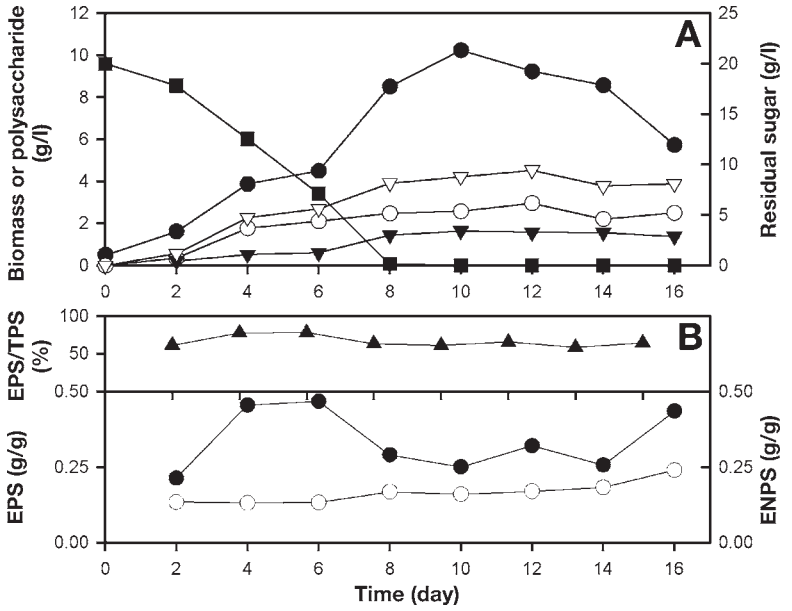

Fig. 2. **(A)** Time profiles of endo- (ENPS) and exopolysaccharide (EPS) production in submerged culture of *Ganoderma lucidum* in a 5-L fermenter. **(B)** Variances in productivities and ratios of the EPS to total polysaccharides (TPS) in a 5-L bioreactor culture. The results were expressed from a single experiment. Symbols for **(A)**: mycelial dry weight (●); EPS (○); ENPS (▼); TPS (▽); Residual sugar (■). Symbols for **(B)**: EPS (●); ENPS (○); EPS/TPS (▼).

Although the EPS production in most submerged cultures of mushrooms was usually higher than ENPS, as observed in this study, higher ENPS production in *G. lucidum* have also been reported *(30)*. These contradictory results imply that the production yields of EPS and ENPS are strongly dependent on strain phenotypes, culture medium, and environments.

In a survey of the literature regarding *G. lucidum* fermentation, the maximum concentrations of EPS established by many investigators are in dispute because of different methods of EPS estimation *(30)*. Some researchers measured it by the phenol-sulfuric method *(46)*, whereas others measured it by the gravimetric method *(47,48)*. For this reason, it is not easy to directly compare the EPS levels achieved from the fermentation of *G. lucidum* established in this work to those reported in the literature.

Two-Stage Batch Fermentation With Controlled pH

Based on the batch experiments without pH control, the two-stage pH operation was performed to further optimize the productions of both mycelial biomass and EPS. Figure 3 shows the time profiles of mycelial growth and EPS production in a 5-L stirred-tank fermenter under different controlled-pH conditions: pH 4.5 during the entire fermentation period, pH shifting from 3.0 to 6.0, or from 6.0 to 3.0 in the middle of exponential

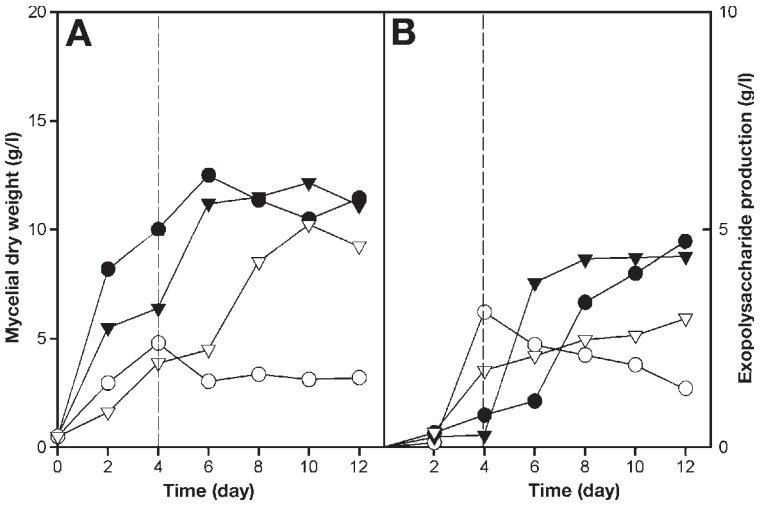

Fig. 3. Time profiles of mycelial growth **(A)** and exopolysaccharides (EPS) **(B)** in submerged culture of *Ganoderma lucidum* under different controlled-pH batch fermentations in a stirred-tank fermenter. Controlled pH shifting from 3.0 to 6.0 (●); controlled pH shifting from 6.0 to 3.0 (○); controlled pH 4.5 (▼); initial pH 4.5 without control (▽).

growth phase (day 4). The fermentation results, particularly the chemical composition and molecular weight of EPS, are listed in Table 1. Among the three pH-control strategies, the maximum mycelial biomass (12.5 g/L at day 6) and EPS production (4.7 g/L at day 12) were achieved when culture pH was shifted from 3.0 to 6.0 at day 4. As expected, EPS production using a tailored two-stage fermentation process (4.7 g/L) was enhanced by 1.6-fold compared to that of the fermentation with an uncontrolled pH of 4.5 (3.0 g/L). Apart from maximum mycelial biomass and EPS concentrations (X_{max} and P_{max}), other values of fermentation parameters were also higher when the pH shifted from 3.0 to 6.0 (Table 2). Although there is no special trend in variation patterns of chemical composition and molecular weight of EPS, it is interesting to note that no top fraction of EPS was obtained from the condition of pH shifting from 3.0 to 6.0 (Table 1).

In the course of two-stage culture, a distinct variance in mycelial morphology was also observed among the three culture conditions (Fig. 4). The typical compact pellets with circular dense cores were formed at pH 4.5, whereas irregular pellets with more dispersed form were observed at controlled pHs shifting from 3.0 to 6.0 and from 6.0 to 3.0. This morphological variance is likely to be related with fermentation efficiency, such as concentrations of mycelial biomass and EPS.

Wagner et al. *(31)* also observed links between morphology and physiology of *G. lucidum*. The authors found that the mycelial pellets originally produced protuberances when glucose was present in the medium, although glucose was not consumed. When the protuberances were liberated into the medium as second-generation pellets, it was then that glucose

Table 1
Carbohydrate Composition and Molecular Weight of Different Exopolysaccharides Produced From Submerged Culture of *Ganoderma lucidum* Under Different pH-Control Processes

Fermenter condition	EPS	Ratio EPS (%)	Carbohydrate composition (%)					*M*w (×10^5 g/mol) (error %)		
			Rib	Xyl	Man	Gal	Glu	Fr-I	Fr-II	Fr-III
pH 4.5	Top	72.0	nd[a]	nd	6.6	nd	93.4	14.79 (0.9)	6.71 (0.3)	0.65 (0.7)
	Bottom	28.0	6.2	4.2	47.4	16.2	26	93.44 (0.6)	0.45 (0.6)	0.28 (0.4)
pH 3.0 → 6.0	Top	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Bottom	100	4.6	nd	23.5	4.9	67	27.55 (1.0)	0.52 (0.3)	0.20 (0.3)
pH 6.0 → 3.0	Top	62.4	nd	nd	19.6	nd	80.4	3.77 (0.9)	0.45 (0.3)	0.20 (0.5)
	Bottom	37.5	2.4	nd	46.4	3.9	47.3	33.18 (1.9)	0.36 (0.4)	0.28 (1.5)

[a]nd, not detected.

Table 2
Fermentation Parameters of the Batch Experiments Under Various pH-Controlled Processes in Submerged Culture of *Ganoderma lucidum* Using a Stirred-Tank Fermenter

Different pH condition		μ^a (d^{-1})	$Q_x{}^b$ (g/L/d)	$Q_p{}^c$ (g/L/d)	X_{max} (g/L)	P_{max} (g/L)	$Y_{p/x}{}^d$ (g/g)	$Y_{x/s}{}^e$ (g/g)	$Y_{p/s}{}^f$ (g/g)
Uncontrolled	pH 4.5	0.148	0.974	0.247	10.24 (10 day)	2.97 (12 day)	0.339	0.437	0.148
Controlled	pH 4.5	0.159	1.783	0.365	12.16 (10 day)	4.38 (12 day)	0.413	0.531	0.219
Controlled:	pH 3.0 → 6.0	0.160	2.000	0.394	12.5 (6 day)	4.73 (12 day)	0.432	0.547	0.234
two-stage	pH 6.0 → 3.0	0.139	0.423	0.112	4.8 (4 day)	3.10 (4 day)	0.496	0.180	0.089

[a]μ (d^{-1}) = (1/X)(dX/dt); [b]Q_x (g/L/d) = (dX/dt); [c]Q_p (g/L/d) = (dP/dt); [d]$Y_{P/X}$ (g/g/d) = (dP/dt)(1/X); [e]$Y_{X/S}$ (g/g/d) = (dX/dt)(1/S); [f]$Y_{P/S}$ (g/g) = (dP/dt)(dS/dt). X, S, and P refer to concentrations of cells, substrate, EPS at a time t, respectively.

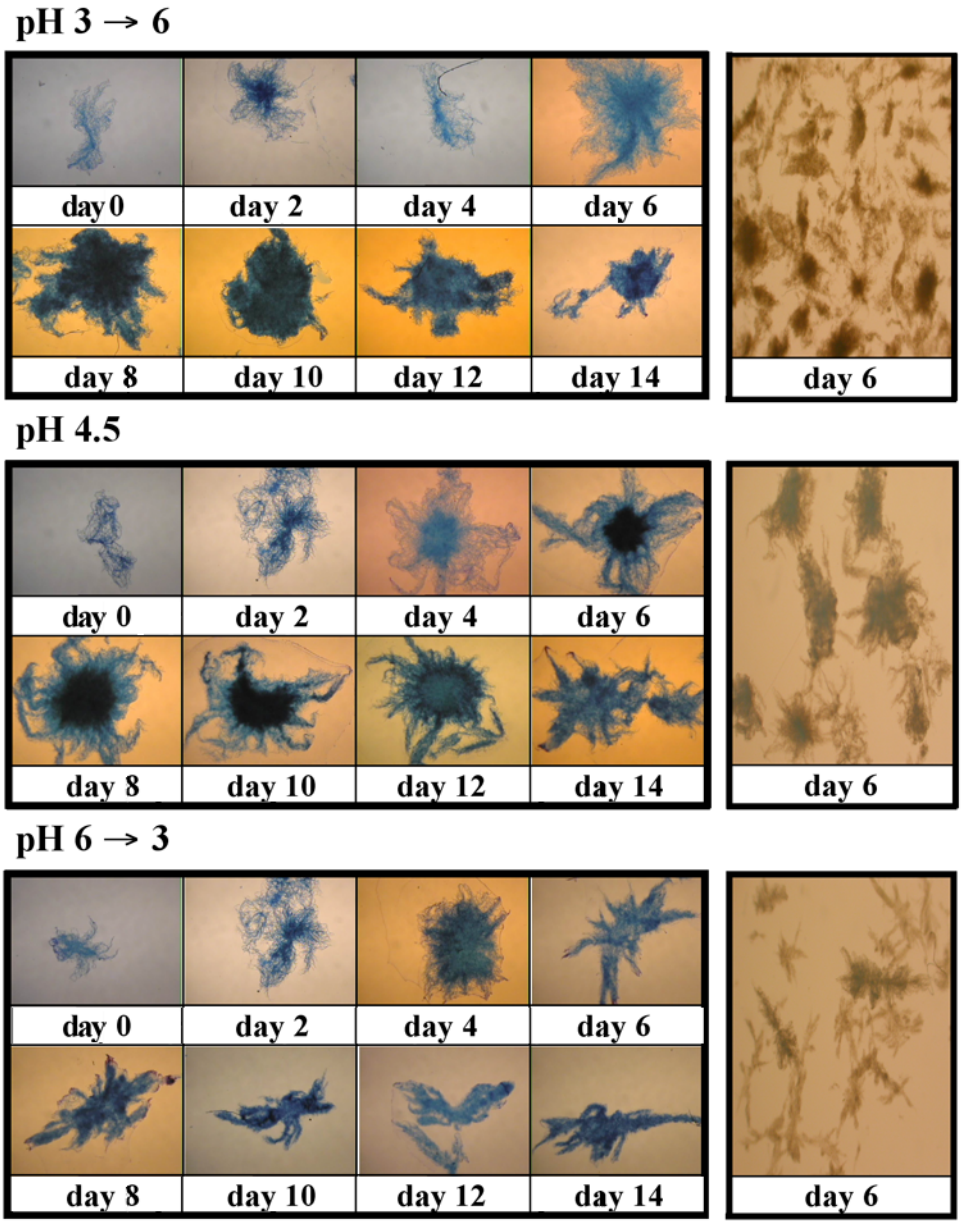

Fig. 4. Time-dependent variation in mycelial morphology during submerged culture of *Ganoderma lucidum* under three different controlled-pH conditions. The magnification ratios of the microphotographs in left panels and right panels are ×32 and ×90, respectively.

consumption began and EPS production accelerated. This relationship between morphology and metabolic event has not been usually found in other investigations (also including this study) concerning *G. lucidum* culture, even though similar nutrients were used.

In our investigation, we found a clear time-dependent variation in morphology in all pH conditions. The mycelial morphology was the most regular form with dense core area under a controlled pH 4.5, which was maintained throughout the entire culture periods. In contrast, larger and more dispersed pellets were observed when the pH was controlled by shifting from 3.0 to 6.0. Also, feather-like pellets were found when the pH was controlled by shifting from 6.0 to 3.0, which is considered to be an undesirable morphological form for EPS production. The more dispersed pellets with high hairiness, as observed by shifted pH from 3.0 to 6.0, seems to be the most favorable morphological form for EPS production in *G. lucidum* submerged culture. Moreover, the numbers of pellets formed were higher when the pH shifted from 3.0 to 6.0, compared to other pH conditions (*see* right panel of Fig. 4).

Similarly, Lee et al. *(26)* also suggested that the maximum production of mycelial biomass and EPS in *G. lucidum* were achieved when culture pHs were controlled at 3.0 and 6.0, respectively. The authors suggested that the bistage pH control technique (shifting the pH from 3.0 to 6.0) at the initial phase of the exponential growth led to enhanced EPS production, but reduced mycelial growth when compared to uncontrolled pH cultures.

However, Fang and Zhong *(33)* described the importance of culture pH in *G. lucidum* fermentation, suggesting that lowering the initial pH from 6.5 to 3.5 led to a higher EPS production. This conflicting finding is likely to be resulted from different physiology of the strains of *Ganoderma* rather than difference in experimental achievements.

Variations in Chemical Composition and Molecular Weight of EPS

A compositional analysis revealed that EPS, produced from uncontrolled pH condition (initial pH 4.5), are heteropolysaccharides consisting mainly of glucose and mannose, whereas ENPS contains mainly glucose (Table 3). Three fractions of polysaccharides (hereafter designated as Fr-I, -II, and -III) were detected by the SEC/MALLS system and their average molecular weights were significantly different, ranging from 1.02 to 56.16 $\times 10^5$ g/mol. In the case of EPS produced from the three pH-controlled conditions, more detailed analysis was carried out with the top and bottom fractions obtained from ethanol precipitation of culture filtrate. It is interesting to note that culture pH strikingly affect the ratio of top and bottom fraction, chemical composition, and molecular weight of EPS (Table 1). Similarly, Shu and Lung *(35)* investigated the effect of pH on the production and molecular distribution of EPS during mycelial culture of the mushroom *Antrodia camphorate*. The authors reported that high-molecular-weight EPS was obtained at lower pH values with lower yields, but low-molecular-weight EPS was obtained at higher pH values with higher yields. In this study, however, there were no distinct patterns in variation of molecular weight of EPS in response to shift of culture pH.

In conclusion, the culture pH in stirred-tank fermentation of *G. lucidum* has a critical effect on the concentrations of mycelial biomass, EPS, chemi-

Table 3
Carbohydrate Composition and Molecular Weight of ENPS and EPS
Produced by Submerged Mycelial Culture of *Ganoderma lucidum* Without pH Control[a]

	Carbohydrate composition (%)					*M*w (×10^5 g/mol) (error %)		
Polysaccharides	Rib	Xyl	Man	Gal	Glu	Fr-I	Fr-II	Fr-III
EPS	0.31	3.99	45.23	6.81	40.87	14.96 (1.0)	3.65 (0.6)	1.02 (0.9)
ENPS	0.79	3.99	9.93	4.15	78.11	56.16 (0.9)	6.65 (0.4)	2.74 (3.0)

[a]Fermentation was carried out for 10 d under initial pH of 4.5 without control using 5-L stirred-tank fermenter.

cal composition, and molecular weight of EPS. Because *G. lucidum* has demonstrated different pH values for optimum mycelial growth and EPS formation, a two-stage pH fermentation strategy has been successfully demonstrated to maximize production of both mycelial biomass (23% increase) and EPS (160% increase) in submerged culture of *G. lucidum*. In conclusion, it should be mentioned that the correlation of process parameters, such as culture pH with product quality, should be carefully considered in fungal fermentation for polysaccharide production.

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