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Enhanced biomass production of *Pycnoporus sanguineus* and alterations in the physiochemical properties of its polysaccharides

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ABSTRACT

The purpose of this research was to physiochemically characterize the expression profiles of polysaccharides produced by *Pycnoporus sanguineus* using different cultural conditions including media utilizing different carbohydrate sources and pH values. Polysaccharides were characterized by size-exclusion chromatography (SEC) and high-performance anion-exchange chromatography (HPAEC). The maximum mycelial growth reached a value of 16.52 ± 1.03 g/l when *P. sanguineus* was fed 20 g/l sucrose with 20 g/l potato dextrose broth (PDB). Medium-high-molecular-weight polysaccharides (50–100 kDa) were largely synthesized by glucose feeding. The synthesis of low-molecular-weight polysaccharides (<30 kDa) decreased when the pH of the medium increased. Fucose, galactose, glucose, and mannose were the dominant sugars in the *P. sanguineus* polysaccharide mixture. We determined correlations between sugar components in the polysaccharides and the type of carbon source in the medium. Feeding with sucrose or glucose resulted in a direct dosage effects on the fucose and mannose components of the polysaccharides.

1. Introduction

Pycnoporus sanguineus, an edible Basidiomycete mushroom, causes white rot of dead hardwoods, is used by indigenous tribes of the Americas and Africa, and has antiviral effects (Smânia et al., 2003). This fungus has long been used in popular medicine of the Americas and Africa for treating a number of illnesses (Perez-Silva, Aguirre-Acosta, & Perez-Amador, 1988). Also, the fungus has been applied to treat wastewater containing heavy metals and an oil-polluted habitat by its biosorption of adsorbs lead, copper, and cadmium properties.

Polysaccharides are of great interest in terms of both ecological and human health aspects. They also play important roles for the fungi themselves as secondary metabolites. There is growing interest in their use as pharmaceuticals. Polysaccharides produced by mushrooms as potentially useful, biologically active ingredients for pharmaceutical uses as anti-inflammation (Lu, Cheng, Lin, & Chang, 2010), anti-hepatitis B surface antigen effect (Lee et al., 2002), immunological activity (Han, Chai, Jia, Han, & Tu, 2010), and anti-tumor activity (Zhang, Cui, Cheung, & Wang, 2007). Obtaining bioactive polysaccharides from cultural of mushrooms will be of great interest for future studies to establish a scaleable method for commercial production. In the middle of the 20th century, improvements in analytical techniques such as chromatography allowed the successful identification of these macromolecules. Although many investigators attempted to obtain optimal submerged-culture conditions for polysaccharide production from several mushrooms, currently available reports on nutritional requirements of cultures are limited to only a few mushrooms, i.e., *Ganoderma lucidum* (Fang & Zhong, 2002) and *Paecilomyces japonica* (Bae et al., 2001). Therefore, it is worthwhile investigating the effects of cultural medium on the production and structural variations of polysaccharides from mushrooms.

Several kinds of mushrooms require starch, sucrose, maltose, glucose, or galactose as carbon sources for submerged culture (Kim et al., 2002). According to Steluti et al. (2004), the fungus of *Botryosphaeria* sp. produces polysaccharides with all of the carbon sources examined, with the highest yields occurring using sucrose, followed by glucose and fructose. Different carbon sources generate similar bioactive polymers with different degrees of branching and distinct polymerization, producing biopolymers that are more or less water-soluble, and as a consequence, may possess higher or lower biological activities (Jin et al., 2003; Zhang, Yang, Ding, & Chen, 1995).

The objectives of this research were to investigate hyphal growth-promoting factors of *P. sanguineus* from media with different carbohydrate sources and initial pH values, and structurally characterize the molecular-weight distribution and sugar com-

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positions of the polysaccharides. The polysaccharides of cultured mycelia from potato-dextrose-broth (PDB), sucrose, and glucose-based media of *P. sanguineus* were investigated. Correlations between polysaccharide structural changes and cultural media were also studied.

2. Materials and methods

P. sanguineus (TFRI1048) was isolated from fruiting body collected from *Cinnamomum* sp. on May 20, 2002.

2.1. Liquid culture

Fungi were maintained on potato dextrose agar (PDA) slants and transferred to fresh medium at 3-week intervals. In each pasteurized Petri dish, 25 ml of PDA medium (39 g/l) was used and incubated at 28 °C for 19 days. The fine mycelia on the media surface were transferred to 800-ml culture flasks containing 100 ml of test medium. Polysaccharides were isolated from 49-day-old cultures. Following incubation, mycelia were rapidly washed with 1 L of 250 mM NaCl during aspiration to remove any contaminating exopolysaccharides. Samples were then lyophilized, and stored at 4 °C, and the dry weight of mycelia was measured.

2.2. Isolation of the polysaccharides

Lyophilized mycelia of the various fungal cultures were extracted twice with 80 °C water in a 1:100 (w/w) ratio for 6 h. The extracts were cooled, and four volumes of 95% ethanol were added, then allowed to precipitate overnight at 4 °C. The precipitated polysaccharides were collected by centrifugation and lyophilized, resulting in a crude brownish polysaccharide sample.

2.3. Size-exclusion chromatography (SEC) of the polysaccharides

A polysaccharide solution in milli-Q water was diluted to give a concentration of 1 mg/ml and was then filtered through a 0.22-μm filter (Millipore, Billerica, MA, USA) before injection onto the SEC column. The flow rate was 0.5 ml/min, with deionized water used as the eluent. A calibration curve was constructed using an authentic standard, Sodex P-82 series (Showa Denko America, Mentor, OH, USA) containing polymaltotriose with molecular weights of 78.8×10^4, 40.4×10^4, 21.2×10^4, 4.73×10^4, and 1.18×10^4 Daltons (Da). The TriSec software program was used to acquire and analyze the Viscotek data. SEC signals were detected using a ViscoTek model TDA-3-1 relative viscometer (Viscotek, Houston, Texas, USA).

2.4. Hydrolysis of the polysaccharides

Acid hydrolysis of the polysaccharides was carried out as follows. One milligram of lyophilized polysaccharides was hydrolyzed with 4.95 N trifluoroacetic acid (TFA) at 80 °C in a heating block for 4 h. The mixture was cooled, evaporated, and then resuspended in milli-Q water.

2.5. Compositional analysis of the polysaccharides

Monosaccharides were separated on an HPAEC system (Dionex BioLC, Sunnyvale, CA, USA) equipped with a gradient pump, a pulsed amperometric detector (PAD-II) with a gold working electrode, and an anion-exchange column (Carbopac PA-10, 4.6 mm × 250 mm, Dionex). Samples were applied using an autosampler (AS3500, SpectraSYSTEM®) via a microinjection valve with a 200-μl sample loop. Monosaccharides were analyzed at an isocratic NaOH (Thermo Fisher Scientific, Waltham, MA, USA)

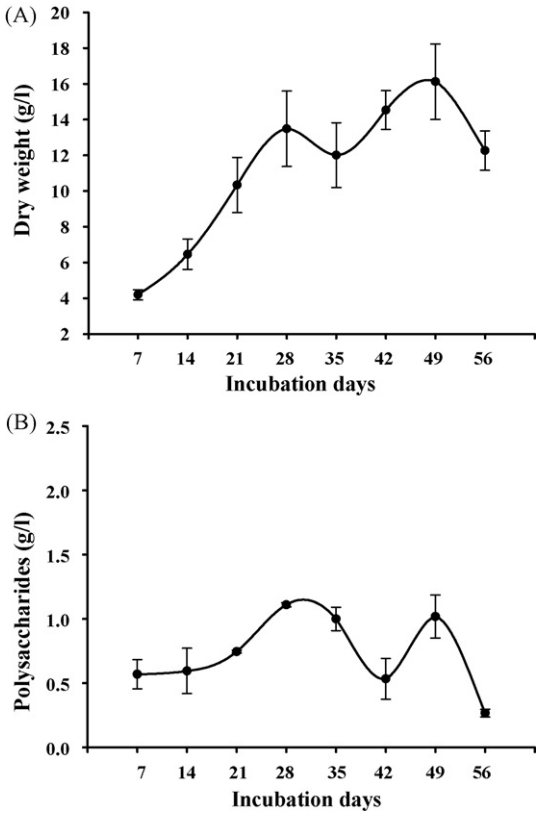

Fig. 1. Time course of (A) growth and (B) polysaccharide production in a mycelial culture of *Pycnoporus sanguineus*. (A) Growth and (B) polysaccharide yields. Data are presented as the mean ± standard error from three independent experiments.

concentration of 18 mM at ambient temperature. Monosaccharides were identified and quantified by comparing them to standards. Data were collected and integrated on a PeakNet system (Dionex).

2.6. Statistical analysis

Data are presented as the mean ± standard error (S.E.), and n represents the number of experiments. In bar graphs, S.E. values are indicated by error bars. Statistical analyses were carried out using Student's unpaired t-tests when applicable. p-values of <0.05 were considered significant.

3. Results and discussion

3.1. Time-course study of growth and polysaccharide production

To maximize the production of fungi, a time-course study was performed on the dry-mass accumulation and yield of polysaccharides. For *P. sanguineus*, the culture period between 7 and 28 days was a linear phase. Beyond 28 days, the culture entered a senescence phase (Fig. 1A). The maximal dry mass accumulated at day 49 at a value of 16.13 ± 2.10 g/l. The time-course study of the polysaccharide yield showed that at 28 days of culture, the highest value of 1.03 ± 0.13 g/l was achieved (Fig. 1B).

3.2. Effects of carbon sources on mycelial growth and polysaccharide production

To evaluate the effects of the carbon source on mycelial growth, the dry biomass was used to measure cell growth. Comparisons were made among sucrose-. glucose-, and PDB-based medium in the dose range of 5–20 g/l of 49-day-cultured mycelia of *P. san-*

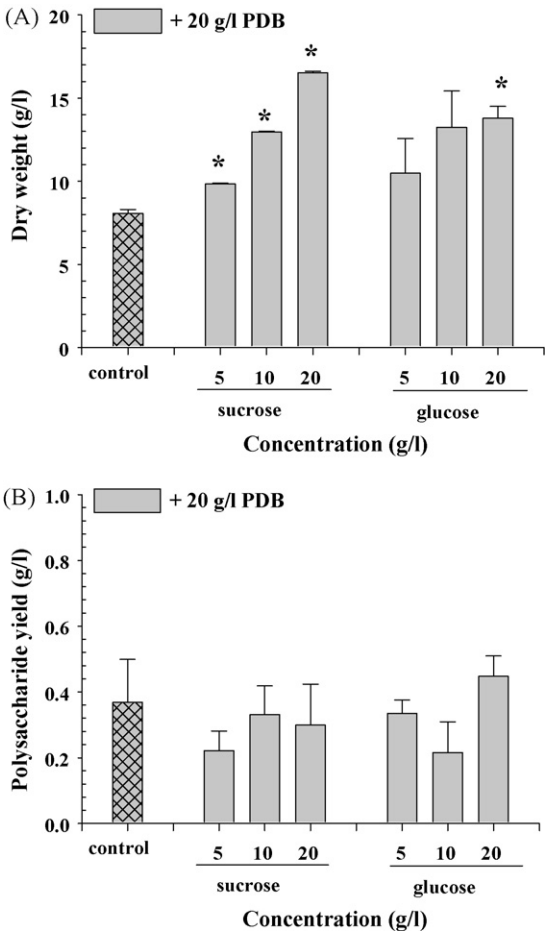

Fig. 2. Effects of basal medium of 20 g/l PDB with sucrose or glucose feeding at doses of 5, 10, and 20 g/l on (A) mycelial growth and (B) polysaccharide yields of *Pycnoporus sanguineus* cultured for 49 days. $p < 0.05$ vs. the control, $n = 3–5$.

guineus (Fig. 2A). Direct dosage effects were shown for sucrose as the carbon source. All sucrose feeding significantly enhanced mycelia growth compared to the control (original medium PDB 20 g/l). The maximum mycelial growth of *P. sanguineus* reached a value of 16.52 ± 1.03 g/l when fed 20 g/l sucrose. On the contrary, the growth of *P. sanguineus* was insensitive to glucose. This may have been due to sucrose playing a balancing role in cell growth due to hydrolysis of invertase and sucrose synthase, and the resulting hexose entering the glycolytic and pentose phosphate pathways (Stepan-Sarkissian & Fowler, 1986).

Each carbon source at the tested dosages was optimized according to the yield of polysaccharides. Comparisons were made among sucrose- and glucose-containing media at the doses of 5, 10, and 20 g/l with 49-day-cultured mycelia of *P. sanguineus* (Fig. 2B). Neither sucrose nor glucose affected polysaccharide production.

Although several kinds of mushrooms frequently require starch, sucrose, maltose, glucose, or galactose as the carbon source for submerged culture (Bae et al., 2001), those results are counter the finding that PDB enhanced the growth of *P. sanguineus* in one study (Winder, 2006). According to Steluti et al. (2004), *Botryosphaeria* sp. produced polysaccharides on all of the carbon sources examined, with the highest yields occurring with sucrose, followed by glucose and fructose. Different carbon sources generate similar bioactive polymers with different degrees of branching and distinct polymerization, producing biopolymers that are more or less water-soluble, and as a consequence, they possess higher or lower biological activities.

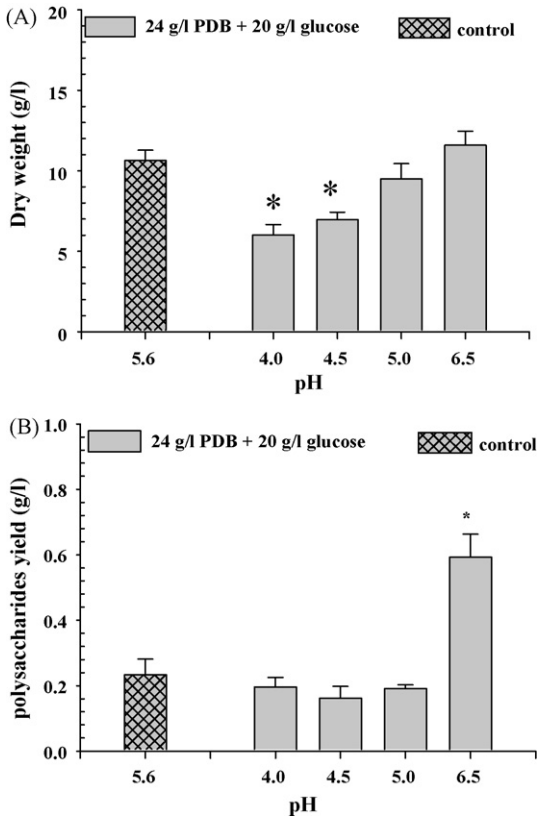

Fig. 3. Effects of the initial pH on (A) mycelial growth and (B) polysaccharide yields of *Pycnoporus sanguineus* cultured for 49 days. $p < 0.05$ vs. the control, $n = 3–5$.

3.3. Effects of the initial pH on mycelial growth and polysaccharide production

To evaluate the effects of the initial pH on mycelial growth, the dry biomass was used to measure cell growth. Comparisons were made among pH 4–6.5 cultures of 49-day-cultured mycelia (Fig. 3A). Medium at pH 5.6 was used as the control. Media at pH 4 and 4.5 showed significantly inhibited mycelial growth compared to that at pH 5.6. A linear trend of mycelial growth was shown as the level of acidity decreased in the medium. The highest growth of *P. sanguineus* was achieved at an initial pH of 6.5 with a value of 11.59 ± 0.87 g/l. The optimal initial pH for other fungal species like *Cordyceps sphecocephala* was reported to be 5.0 (Oh, Cho, Nam, Choi, & Yun, 2007).

To evaluate the effects of the initial pH on polysaccharide production, comparisons were made among pH 4–6.5 of 49-day-cultured mycelia of *P. sanguineus* (Fig. 3B). Medium at pH 5.6 was used as the control. For *P. sanguineus*, medium at pH 6.5 showed significantly enhanced polysaccharide production with a value of 0.59 ± 0.07 g/l.

3.4. Effects of carbon sources on the molecular-weight distribution of polysaccharides from P. sanguineus

To investigate the effects of carbohydrate-based media on structural variations of fungal polysaccharides, the polysaccharides were characterized according to their molecular size distributions and sugar compositions. A calibration curve was constructed using a series of standards containing polymaltotriose with molecular weights of 788, 404, 212, 112, 47.3, 22.8,11.8, and 5.9 kDa. A regression equation was determined by the log [Mw] (Y) and the fraction number (X) as $Y = 9.34 - 0.24X$, $R^2 = 0.99419$. The molecular-weight distribution of the lyophilized polysaccharide-

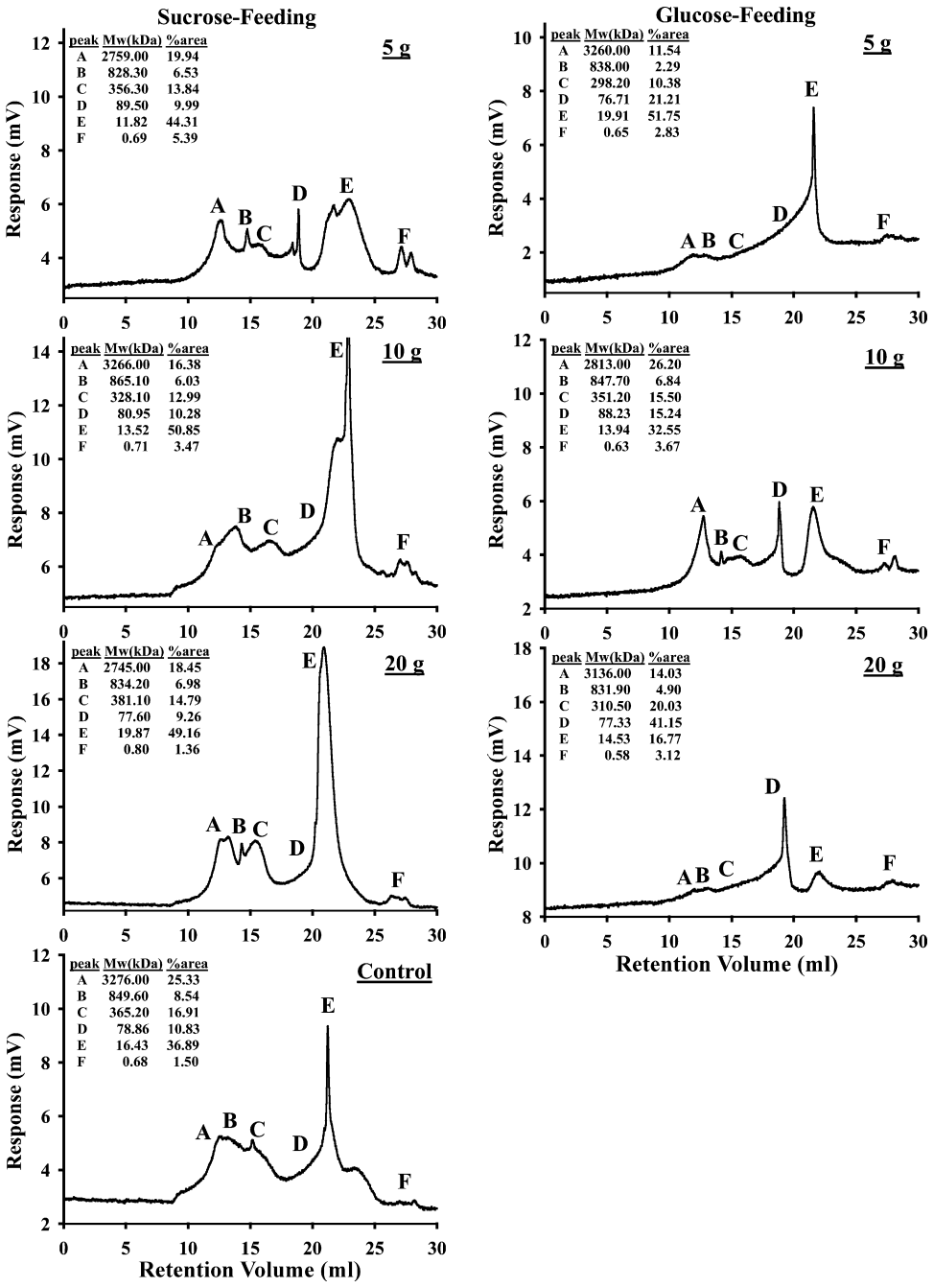

Fig. 4. Effects of basal medium of 20 g/l PDB with sucrose or glucose feeding at doses of 5, 10, and 20 g/l on the molecular-weight distribution of polysaccharides from *Pycnoporus sanguineus*. Polysaccharides were isolated from 49-day-old mycelia cultured in media with different carbon sources.

containing preparation was chromatographed and characterized as very-high- (>1000 kDa, denoted peak A), high- (100–1000 kDa, denoted peaks B and C), medium-high- (50–100 kDa, denoted peak D), low- (1–20 kDa, denoted peak E), and very-low-molecular-weight polysaccharides (<1 kDa, denoted peak F) (Fig. 4).

To investigate the effects of different dosages of the same carbon source, we examined if the molecular-weight distribution contained similar polysaccharide polymers and also if different carbon sources generated different polysaccharide profiles. The results showed that when fed with sucrose or glucose, mycelia generated different polysaccharide patterns from that of control medium. The same carbon source of sucrose in the dose range of 10–20 g/l generated similar polysaccharide profiles, and at <10 g/l, fewer low-molecular-weight polysaccharides (peak E) were synthesized. Different carbon sources generated different polysaccharide profiles; medium-high-molecular-weight (peak D) polysaccharides were largely synthesized by glucose feeding (Fig. 4).

3.5. Initial pH effects on the molecular-weight distribution of polysaccharides from P. sanguineus

To elucidate the initial pH effect on the structure of polysaccharides, comparisons were made among culture media at pH 4.0–6.5. After chromatography, the molecular-weight distribution of lyophilized polysaccharide-containing preparations was characterized as very-high- (> 1000 kDa, denoted peak A), high- (100–500 kDa, denoted peak B), medium-high- (500–30 kDa, denoted peaks C, D, and E), and low-molecular weight (<30 kDa,

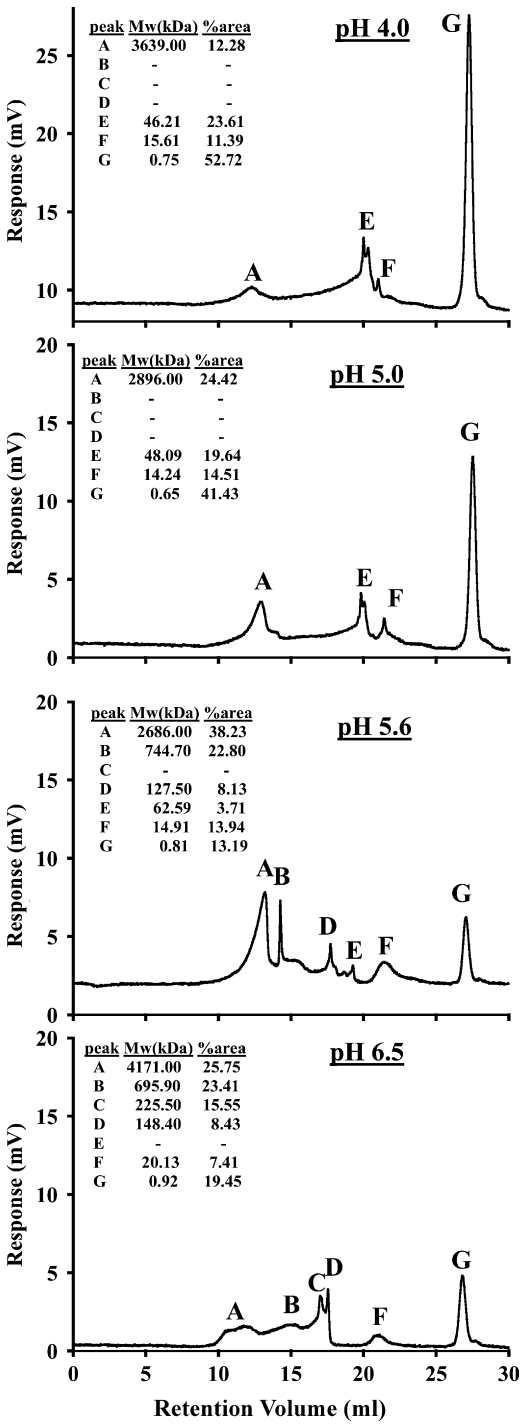

Fig. 5. Initial pH effects on the molecular-weight distribution of polysaccharides from *Pycnoporus sanguineus*. Polysaccharides were isolated from 49-day-old mycelia cultured in media with different carbon sources.

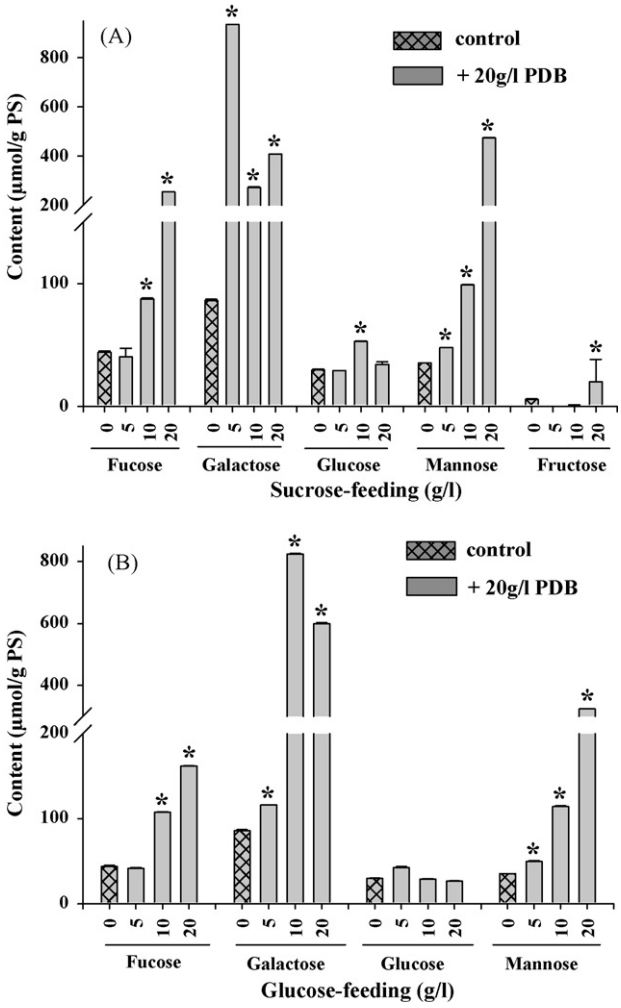

Fig. 6. Effects of the carbon source on the sugar composition of polysaccharides of *Pycnoporus sanguineus*. $^*p < 0.05$ vs. the control, $n = 4$.

denoted peaks F and G) (Fig. 5). The results showed that in medium at pH 5.6, the maximal synthesis of very-high-molecular-weight polysaccharide of peak A was achieved. The high-molecular-weight polysaccharide of peak B was newly synthesized when the initial pH was >5.6, and medium-high-molecular-weight polysaccharides of peaks C, D, and E decreased when the pH of the medium increased. The synthesis of low-molecular-weight polysaccharide of peak G decreased when the pH of the medium increased.

In nature, polysaccharides occur as mixtures of heterogeneous cellular components (Kim et al., 2003); it is first necessary to isolate, purify, and structurally characterize these polysaccharides. It was reported that the carbon source in the culture medium of *Botryospaeria rhodina* affected the side-chain structures of polysaccharides but not the main-chain profile. Sucrose produced less branching (21%) than fructose (31%) (da Silvaa et al., 2005). In addition to the carbon source, the culture pH also affected the polysaccharide production of edible mushrooms of *Grifola frondosa* (Lee et al., 2004). A comparison of polysaccharide production by *B. rhodina* on several carbohydrate carbon sources was recently reported (Steluti et al., 2004). The highest yields of polysaccharides occurred with sucrose followed by glucose and fructose. According to Jin et al. (2003), different carbon sources for polysaccharides produced similar bioactive polymers with different degrees of branching and distinct polymerization, different water solubilities, and different biological activities.

3.6. Compositional analysis of polysaccharides

A compositional analysis was performed after the polysaccharide fraction was completely hydrolyzed, and the carbohydrate components are presented. Fucose, galactose, glucose, and mannose were the dominant sugars in the *P. sanguineus* polysaccharide mixture (Figs. 6 and 7). Feeding with sucrose or glucose resulted in a direct dosage effect on the fucose and mannose components of the polysaccharides (Fig. 6). The effect of the initial pH on the sugar composition of the polysaccharides was investigated, and compar-

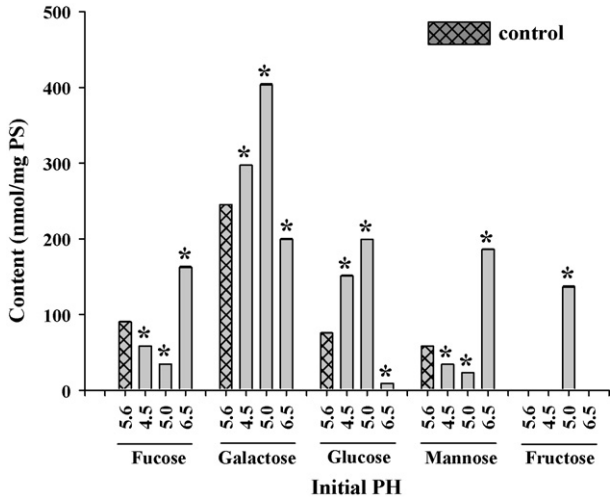

Fig. 7. Effects of the initial pH on the sugar composition of polysaccharides of *Pycnoporus sanguineus*. $^{*}p < 0.05$ vs. the control, $n = 4$.

isons were made among cultured media at pH 4.0–6.5. Medium at pH 5.6 was used as the control. All tested pH levels showed inhibition of the fucose and mannose contents except for medium at pH 6.5. Increasing the pH from 4.0 to 5.0 resulted in a direct effect on the galactose and glucose components of the polysaccharides. Fructose only appeared when the medium was at pH 5.0 (Fig. 7).

4. Conclusions

The present study demonstrates an efficient strategy for optimizing medium components for maximum mycelial growth at a value of 16.52 ± 1.03 g/l fed of 20 g/l sucrose with 20 g/l PDB for *P. sanguineus*. The above results will be useful for the large-scale cultivation of *P. sanguineus*. In this study, we characterized fungal polysaccharides using actual physiochemical evidence. Two important findings were that different carbon sources and initial pH values generated different polysaccharide profiles and chemical compositions. Medium-high-molecular-weight (50–100 kDa) polysaccharides were largely synthesized by glucose feeding. High-molecular-weight polysaccharides (100–500 kDa) were newly synthesized when the initial pH was >5.6. The composition of polysaccharides depended on the sugar used as the carbon source. Future study will be performed to elucidate the structure of the specific polysaccharide fraction.

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