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Promotion of fungal growth and underlying physiochemical changes of polysaccharides in *Rigidoporus ulmarius*, an edible Basidiomycete mushroom

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ABSTRACT

Rigidoporus ulmarius is an edible fungus used for medicinal purposes. The aim of this research was to investigate the effects of different carbon sources and initial pH values of the medium on mycelial growth, and analyze underlying changes in polysaccharides. The maximum mycelial growth of 20.91 g/l was obtained at 20 g/l potato dextrose broth (PDB) with 20 g/l glucose, pH 4.5. Increasing sucrose- or glucose concentration from 5 to 20 g/l in the media enhanced mycelia synthesizing polysaccharides in the molecular-weight range (>600 kDa), and inhibited that of polysaccharide in molecular-weight range (5–20 kDa). When the initial pH of the medium increased from 4.5 to 6.5, mycelia synthesized polysaccharides in high-molecular-weight (>600 kDa) decreased and that of low-molecular weight (8–11 kDa) polysaccharides increased. Feeding with sucrose or glucose resulted in a direct dosage effect to the fucose, glucose, and mannose components of the polysaccharides.

1. Introduction

Fungal polysaccharides are of great interest from ecological aspects and because of human health demands. They also play important roles for the fungi themselves as primary metabolites. There is growing interest in their use as pharmaceuticals due to their unique physiological activities (Huang, Jin, Zhang, Cheung, & Kennedy, 2007; Zhao, Dong, Chen, & Hu, 2010; Zhu, Chen, & Lin, 2007). In the middle of the 20th century, improvements in analytical techniques such as chromatography allowed the successful identification of these macro-molecules.

The mushroom, *Rigidoporus ulmarius*, of the Polyporaceae, found mainly on broadleaf trees, is used as an edible fungus for medicinal purposes (Boa, 2004). Our previous study showed that the polysaccharides exhibited antiangiogenic activities and the ethanolic extract had anti-inflammatory properties (Chen, Lu, Cheng, & Wang, 2005; Cheng et al., 2009). It is worthwhile boosting production from a culture system in a scaleable method for pharmaceutical applications. For obtaining bioactive polysaccharides from mushrooms, submerged culture has obvious potential for higher mycelial production in a compact space and a shorter time with fewer chances for contamination. Many investigators attempted to determine optimal submerged culture conditions for polysaccharide production from several mushrooms (Bae, Sinha, Park, Song, & Yun, 2000; Fang & Zhong, 2002). During submerged culture, many mushrooms utilize glucose, galactose, sucrose, maltose, or starch as carbon sources (Bae et al., 2001; Kim et al., 2002). Several kinds of edible and medicinal mushrooms that produce polysaccharides were influenced by the environmental factors (Lin & Sung, 2006). 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). Therefore, it is worthwhile investigating the effects of carbon sources on structural variations of polysaccharides produced during cultivation.

In this study, we attempted to elevate the total dry weight and polysaccharide production from the culturing system, and determine the correlation between polysaccharide structural changes and cultural media with different carbohydrate sources and initial pH values.

2. Materials and methods

R. ulmarius isolate #63 (TFRI1058) was isolated from fruiting bodies collected from northern Taiwan (Fu-Shan Research Station, Ilan County) in February 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 sterile Petri dish, 25 ml of PDA medium (39 g/l) was used and incubated at 28 °C for 19 days (Cheng et al., 2009). For liquid culture,

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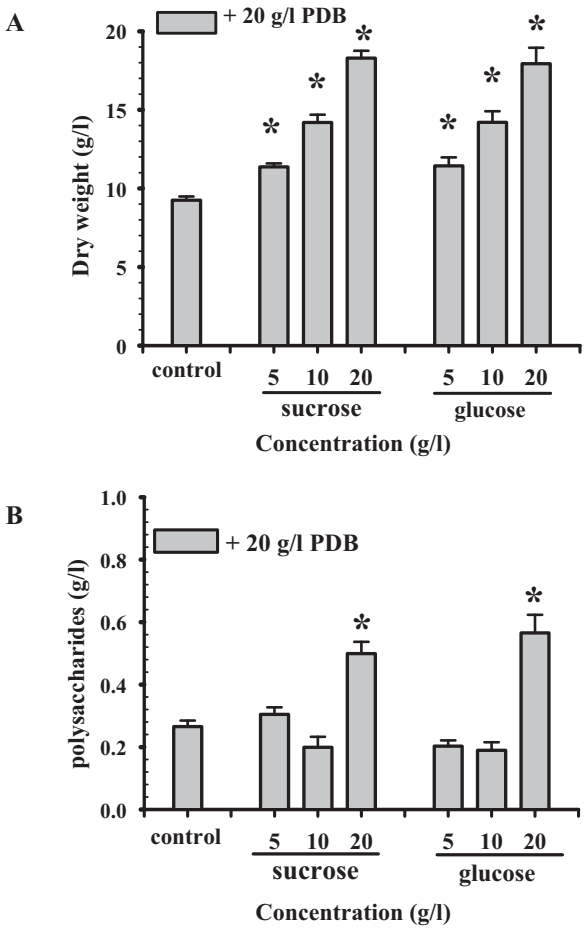

Fig. 1. Effects of basal medium of 20 g/l PDB with sucrose or glucose feeding at 5, 10, and 20 g/l on (A) mycelial growth and (B) polysaccharide yields of *R. ulmarius* cultured for 49 days. $p < 0.05$ vs. the control, $n = 3–5$.

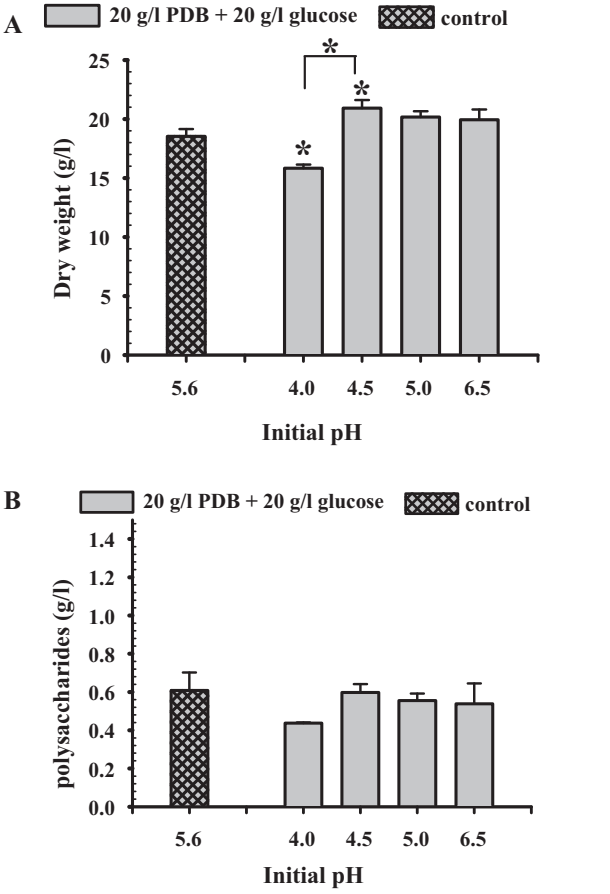

Fig. 2. Effects of the initial pH on (A) mycelial growth and (B) polysaccharide yields of *R. ulmarius* cultured for 49 days. $p < 0.05$ vs. the control, $n = 3–5$.

19-day-old hyphae of *R. ulmarius* were inoculated into an 800-ml wide-base culture flask containing 100 ml of 20 g/l potato dextrose broth (PDB; Difco™, BD Diagnostic Systems, Sparks, MD, USA) as the basal medium (control), with evaluated media containing sucrose or glucose at test concentrations of 5–20 g/l and pH 5.6 except as specified. The size of the inoculum was about 80 mm in diameter. Culture flasks were kept still, and were incubated at 28 °C. 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 contaminating exopolysaccharides. Samples were then lyophilized and stored at 4 °C, and the dry weight of the mycelia was measured.

2.2. Isolation of polysaccharides

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 contaminating exopolysaccharides. Samples were then lyophilized and stored at 4 °C, and the dry weight of the mycelia was measured. Lyophilized mycelia of the various fungi 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 polysaccharides

A polysaccharide solution in milli-Q water was diluted to give a concentration of 1 mg/ml and was then passed through a 0.22-μm filter (Millipore, Billerica, MA, USA) before being injected into 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 signal detection was performed using a ViscoTek model TDA-3-1 relative viscometer (Viscotek).

2.4. Hydrolysis of 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 a high-performance anion exchange chromatography (HPAEC) system (Dionex BioLC, Sunnyvale, CA, USA) equipped with a gradient pump, a pulsed amperometric detector (PAD-II) using a gold working electrode, and an anion-exchange column (Carbopac PA-10, 4.6 mm × 250 mm,

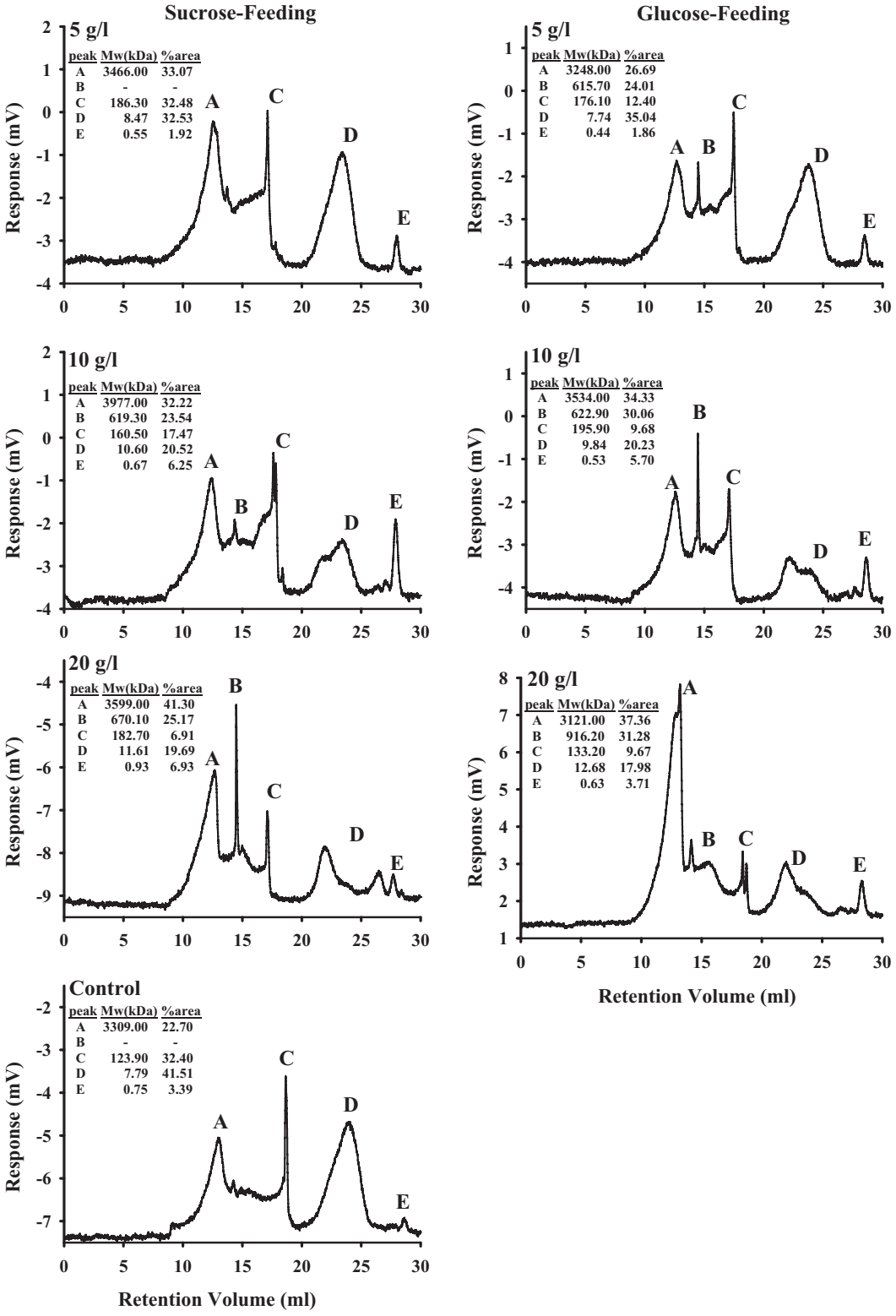

Fig. 3. Effects of basal medium of 20 g/l PDB with sucrose or glucose feeding at 5, 10, and 20 g/l, pH 5.6 on the molecular weight distribution of polysaccharides from *R. ulmarius*. Polysaccharides were isolated from 49-day-old mycelia cultured in media with different carbon sources.

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) concentration of 18 mM at ambient temperature. Monosaccharides were identified and quantified by comparison 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. Effects of carbon sources on mycelial growth and polysaccharide production

To evaluate the effects of different carbon sources on the mycelial growth of *R. ulmarius*, sucrose or glucose was added in the dose range of 5–20 g/l on 20 g/l PDB basal medium (control) for 49 days (Fig. 1A). A direct dosage effect was shown with sucrose as the carbon source for *R. ulmarius*. All treatments with the addition of sucrose or glucose showed significantly enhanced mycelia growth compared to the control. This suggests that fungal growth of *R. ulmarius* was sensitive to both sucrose and glucose. This could be due to sucrose playing a balancing role in cell growth through hydrolysis by invertase and sucrose synthase, and the resultant hexose enters the glycolytic and pentose phosphate pathways (Stepan-Sarkissian & Fowler, 1986). Tang, Zhu, Li, Mi, and Li (2008) reported that during the submerged fermentation of the mushroom, *Tuber sinense* (Chinese truffle), in shaking flasks, sucrose, glucose, and fructose were identified as the three best carbon sources for mycelial growth and polysaccharide production.

To evaluate the effects of carbon sources on the biosynthesis of polysaccharides, polysaccharides were extracted from 49-day-cultured mycelia of *R. ulmarius* (Fig. 1B). Sucrose or glucose at a level of 20 g/l significantly enhanced production of polysaccharides. The results suggest that an increase in the carbon source increased polysaccharide production. According to Steluti et al. (2004), the fungus *Botryosphaeria* sp. produced polysaccharides on all of the carbon sources examined, with the highest yields occurring with sucrose followed by glucose and fructose. Polysaccharides, which are defined as secondary metabolites, seemed to be able to be produced under overflow metabolism.

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

To evaluate the effects of the initial pH on mycelial growth, *R. ulmarius* was cultivated in basal medium at different initial pH values (4.0–6.5) (Fig. 2). For *R. ulmarius*, the extreme acidity of the initial pH 4 significantly inhibited mycelial growth, and mild acidity of pH 4.5 showed the highest growth of *R. ulmarius* with a value of 20.91 ± 0.69 g/l (Fig. 2A). It was reported that the optimal initial pH was pH 5.0 for *Cordyceps sphecocephala* (Oh, Cho, Nam, Choi, & Yun, 2007) and pH 6.5 for *Ganoderma lucidum* (Chang, Tsai, & Houng, 2006).

To evaluate the effects of the initial pH on polysaccharide production, comparisons were made among 49-day-cultured mycelia of *R. ulmarius* at pH 4–6.5 (Fig. 2B). No significant differences were found in polysaccharide production among all tested pH values. The fact that the highest growth of *R. ulmarius* occurred at an initial pH 4.5 did not result in high yields of polysaccharides. Similar results were reported that rapid mycelial growth was not correlated with high production yields of exopolysaccharides in *Antrodia cinnamomea* (Lin & Sung, 2006).

3.3. Effects of carbon sources on the molecular weight distribution of polysaccharides from R. ulmarius

To investigate the effects of carbohydrate-based media on structural variations of fungal polysaccharides, 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 formulated between the log[Mw] (Y) and the fraction number (X) as $Y = 9.34 - 0.24X$, $R^2 = 0.99419$. The molecular

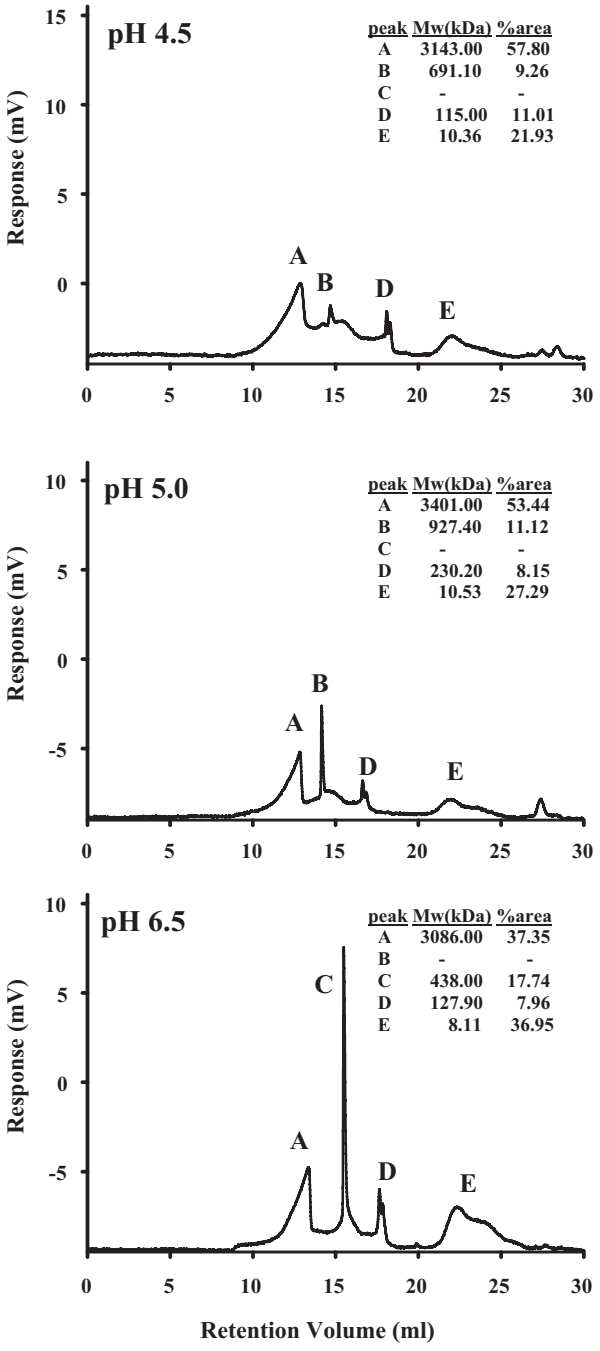

Fig. 4. Initial pH effects on the molecular weight distribution of polysaccharides from *R. ulmarius*. Polysaccharides were isolated from 49-day-old mycelia cultured on 20 g/l PDB, 20 g/l glucose media with different initial pH.

weight distribution of the lyophilized polysaccharide-containing preparation was chromatographed and characterized as very-high- (>1000 kDa, denoted peak A), high- (600–1000 kDa, denoted peak B), medium- (100–200 kDa, denoted peak C), low- (8–11 kDa, denoted peaks D), and very-low-molecular-weight polysaccharides (<1 kDa, denoted peak E) (Fig. 3).

Comparisons were made among control and sucrose- or glucose-fed mycelial polysaccharides. The initial pH value of the media while supplementing sucrose and glucose was 5.6. The major polysaccharide population of the control was low-molecular-weight polysaccharides (peak D) in the percent area of 41.51. The higher sucrose or glucose in the media, the lower percent area of peak D was synthesized. The very-high-molecular-weight

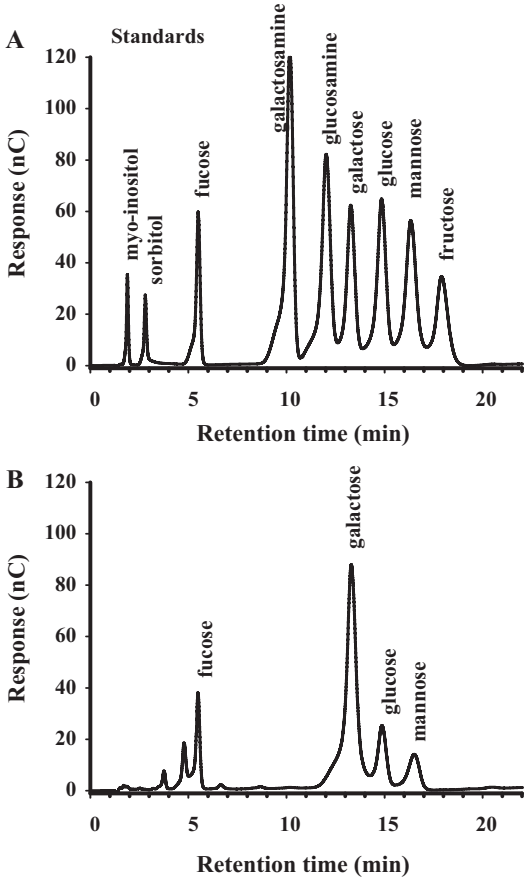

Fig. 5. High-performance anion-exchange chromatography (HPAEC) of *R. ulmarius* polysaccharide hydrolysates. (A) Monosaccharide standards; (B) typical chromatogram of polysaccharide hydrolysates of *R. ulmarius*. The HPAEC analysis was carried out in 18 mM NaOH for 22 min at ambient temperature.

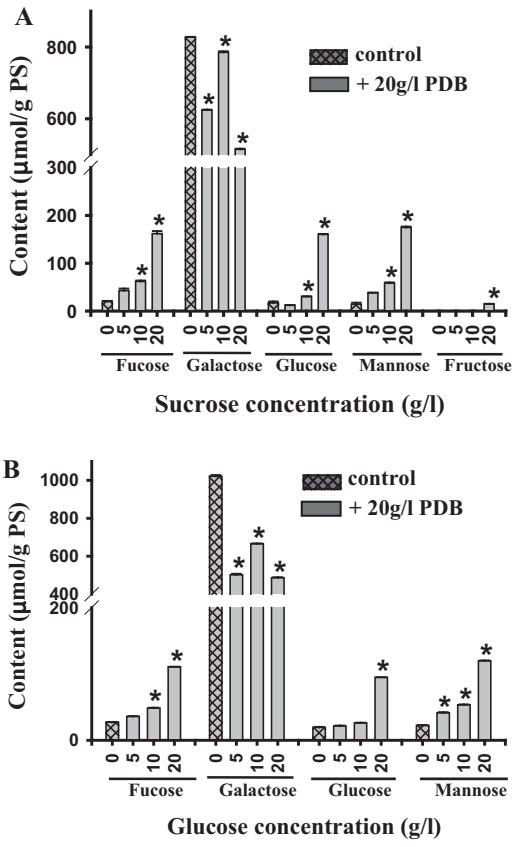

Fig. 6. Effects of carbon source on the sugar composition of polysaccharides of *R. ulmarius*. $^{*}p < 0.05$ vs. the control, $n = 4$.

polysaccharides (peak A) and high-molecular-weight polysaccharides (peak B) were increased with the feeding with sucrose or glucose. To address the effects of different dosages of the same carbon source, we examined if they generated similar polysaccharide polymers according to the molecular weight distribution, and also if they generated different polysaccharide profiles with different carbon sources. The results showed that when fed with sucrose or glucose, mycelia generated similar polysaccharide patterns. Sucrose- or glucose-fed mycelia in the concentration of 20 g/l resulted in higher proportions of both very-high- (peak A) being synthesized in the percent area of 41.30 and 37.36, respectively. Sucrose- or glucose-fed mycelia in the concentration of 5 g/l resulted in higher proportions of low-molecular-weight polysaccharides (peak D) being synthesized in the percent area of 32.53 and 35.04, respectively. The results suggest that an increase in the carbon source increased the proportion of polysaccharides of high molecular weight. An excess amount of the carbon source may have been a great advantage to drive the biosynthetic pathway of polysaccharides to highly complex molecules. Depletion of the carbon source may have resulted in low-molecular-weight polysaccharides being synthesized.

3.4. Initial pH effects on the molecular weight distribution of polysaccharides from R. ulmarius

To elucidate effects of the initial pH of the 20 g/l PDB and 20 g/l glucose medium on the structure of polysaccharides isolated from cultured *R. ulmarius*, polysaccharides were characterized according to their molecular weight distributions. The molecular weight distribution of the lyophilized polysaccharide-containing preparation was chromatographed and characterized as very-high- (>1000 kDa, denoted peak A), high- (600–1000 kDa, denoted peak B), medium- (300–500 kDa, denoted peak C), low- (100–300 kDa, denoted peak D), and very-low-molecular-weight polysaccharides (8–11 kDa, denoted peak E) (Fig. 4). The results showed that the synthesis of very-high-molecular weight polysaccharides of peak A decreased as the initial pH of the medium increased. Also, high-molecular-weight polysaccharides of peak B shifted to medium-molecular-weight of peak C with an increase in the initial pH. The synthesis of very-low-molecular-weight polysaccharide of peak E increased when the initial pH increased. The results showed that growing mycelia in an acid-stressed condition might steer them toward the synthesis of high-molecular-weight polysaccharides.

3.5. Compositional analysis of polysaccharides

A compositional analysis was performed after the polysaccharide fraction was completely hydrolyzed, and the chemical profile is shown in Fig. 5. Fucose, galactose, glucose, and mannose were the dominant sugars in the polysaccharide mixture (Figs. 6 and 7). Increasing the sucrose or glucose concentration in the media resulted in a direct dosage effect on the fucose, glucose, and mannose components of the polysaccharides (Fig. 6). Fructose was only detected in sucrose-fed mycelia. To investigate the effects of the initial pH on the sugar composition of the polysaccharides, comparisons were made among cultured media from pH 4.5 to 6.5, and medium with pH 5.6 was used as a control. All tested pH values showed inhibition of the fucose and mannose contents (Fig. 7). These results suggest that different structures of polysaccharides formed depending on the kind of carbon source used.

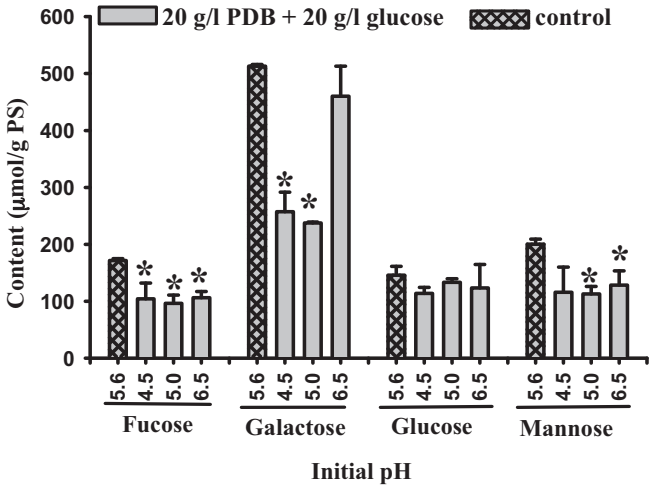

Fig. 7. Effect of initial pH on the sugar composition of polysaccharides of *R. ulmarius*. $^{*}p < 0.05$ vs. the control, $n = 4$.

4. Conclusions

In conclusion, the present study demonstrated an efficient strategy for optimizing the medium and initial pH for mycelial growth of *R. ulmarius*. Increasing sucrose- or glucose concentration in the media enhanced mycelia synthesizing polysaccharides in the high-molecular-weight range (>600 kDa). When the initial pH of the medium increased, mycelia synthesized polysaccharides in low-molecular weight (8–11 kDa) increased. Presumably, high amount of carbon source may have been a great advantage to drive the biosynthetic pathway of polysaccharides to highly complex molecules. The composition of the polysaccharides depended on the kind and dosage of the carbon source used. The information about composition and molecular weight distribution of resultant polysaccharide which constitute the base of important class of microbial polysaccharides, is of great significance for applied studies, the design and synthesis of biologically active substances.

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