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Enhanced Production of Exopolysaccharides by Fed-batch Culture of *Ganoderma resinaceum* DG-6556

Hyun Mi Kim[1], Soon-Young Paik[2], Kyung Soo Ra[3], Kwang Bon Koo[4], Jong Won Yun[1] and Jang Won Choi[4,*]

[1]*Department of Biotechnology, College of Engineering, Daegu University, Kyungsan, Kyungbuk 712-714, Republic of Korea*
[2]*Department of Microbiology, College of Medicine, The Catholic University of Korea, Seoul 137-701, Republic of Korea*
[3]*Department of Food and Nutrition, Daegu Technical College, Daegu 704-721, Republic of Korea*
[4]*Department of Bioindustry, College of Life & Environment, Daegu University, Kyungbuk 712-714, Republic of Korea*

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The objectives of this study were to optimize submerged culture conditions of a new fungal isolate, *Ganorderma resinaceum*, and to enhance the production of bioactive mycelial biomass and exopolysaccharides (EPS) by fed-batch culture. The maximum mycelial growth and EPS production in batch culture were achieved in a medium containing 10 g/l glucose, 8 g/l soy peptone, and 5 mM $MnCl_2$ at an initial pH 6.0 and temperature 31°C. After optimization of culture medium and environmental conditions in batch cultures, a fed-batch culture strategy was employed to enhance production of mycelial biomass and EPS. Five different EPS with molecular weights ranging from 53,000 to 5,257,000 g/mole were obtained from either top or bottom fractions of ethanol precipitate of culture filtrate. A fed-batch culture of *G. resinaceum* led to enhanced production of both mycelial biomass and EPS. The maximum concentrations of mycelial biomass (42.2 g/l) and EPS (4.6 g/l) were obtained when 50 g/l of glucose was fed at day 6 into an initial 10 g/l of glucose medium. It may be worth attempting with other mushroom fermentation processes for enhanced production of mushroom polysaccharides, particularly those with industrial potential.

Keywords: exopolysaccharides, fed-batch culture, mushrooms, submerged culture, *Ganoderma resinaceum*

Ganoderma are basidiomycete fungi belonging to the order Polyporales, and the family Ganodermataceae. Among the numerous species of *Ganoderma*, the most widely used one is *G. lucidum* (Lee *et al*., 2006). It serves as a traditional medicine in Asian countries, due to its diverse biological activities, which include anti-cancer (Ooi and Liu, 2000; Cao and Lin, 2004), anti-allergy (Tasaka *et al*., 1988), anti-inflammatory (Lin *et al*., 1993; Mizuno *et al*., 1995; Liu *et al*., 2003), antioxidant (Ng *et al*., 1993; Lee *et al*., 2001; Lakshmi *et al*., 2003), blood pressure reduction (Kanmatsuse *et al*., 1985; Kabir *et al*., 1988; Lee and Rhee, 1990), hypoglycemic effects (Yang *et al*., 2002; Yang *et al*., 2004; Zhang and Lin, 2004), alpha-glucosidase inhibitor (Kim and Nho, 2004) and platelet-aggregation-inhibition (Shimizu *et al*., 1985; Tang and Zhong, 2002). More specifically, *G. lucidum* produces oxygenated triterpenes which are cytotoxic to hepatoma cells, and act to inhibit histamine and thrombin-induced platelet aggregation (Shiao *et al*., 1994; Wu *et al*., 2001).

Many investigators have tried to cultivate mushrooms on solid artificial media for fruit body formation in order to obtain polysaccharides (Bae *et al*., 2000; Bhargava *et al*., 2002). However, this method does not guarantee a standardized product, since composition of bioactive substituents varies from batch to batch. As a result, attention has been paid to the use of submerged culture for the production of mycelial biomass and bioactive polysaccharides (Kawagoe *et al*., 1999; Lee *et al*., 1999; Park *et al*., 2001). Many investigators have demonstrated the various optimum submerged culture conditions for *G. lucidum* and their production of mycelial biomass and exopolysaccharides (EPS) (Wagner *et al*., 2003). However there are no reports describing submerged culture conditions of *G. resinaceum* even though it is expected to have similar

* To whom correspondence should be addressed.
(Tel) 82-53-850-6756; (Fax) 82-53-850-6769
(E-mail) chjawo @daegu.ac.kr

biological activities to *G. lucidum*.

In the present study, the optimum submerged culture conditions for a new fungal isolate of *G. resinaceum* were determined for the production of mycelial biomass and exopolysaccharides (EPS). In addition, a process for the efficient production of both mycelial biomass and EPS using fed-batch fermentation was developed.

Materials and Methods

Isolation and identification of the fungus

The fungus was isolated from the gills of *G. resinaceum* in Shabsheer El-Hassa village, El-Garbiha governorate, Egypt. The isolated strain was phylogenetically identified by ITS-5.8S rDNA sequencing analysis. The chromosomal DNA of the strain was isolated from fresh mycelium using a Wizard genomic DNA purification kit (Promega, USA) according to the manufacturer's protocol. The resulting genomic DNA was amplified using *Taq* polymerase (Applied Biosystem, USA), and the primers ITS1 (5'-TCCGTA GGTGAACCTGCGG-3') and ITS4R [5'- CAGACTT(G/A) TA(C/T)ATGGTCC AG-3'] (Carbone and Kohn, 1993) on a Techne gene thermocycler (GMI Inc., USA) under the following conditions: 95°C-5 min, 45°C-1 min, 72°C-2 min (1 cycle); 95°C-1 min, 45°C-30 sec, 72°C-2 min (29 cycle); 72°C-10 min (1 cycle). The PCR products were purified using Wizard SV Gel and PCR clean-up system (Promega, USA). The resulting products were cloned into the pGEM-Teasy vector (Promega, USA) and sequenced in both directions using M13 forward and reverse primers with an automated DNA sequencer (ABI PRISM® 3700, Applied Biosystems, USA). The nucleotide sequence obtained was compared with those of GenBank using the NCBI Blast program. Sequence homology was comparatively analyzed using the Clustal X program (Thompson *et al*., 1994). In the end, the sequence of the ribosomal RNA gene from the isolated strain was identified as that of *G. resinaceum* (accession number : AF248334S2) and named DG-6556 (GenBank data homology search result >99%, data not shown).

Microorganism and media

A culture of *G. resinaceum* was maintained on potato dextrose agar (PDA) slants stored at 4°C and subcultured every 4 weeks. The seed cultures were grown in 250 ml flasks containing 50 ml of MCM medium (Mushroom Complete Medium; 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 days.

Inoculum preparation and flask cultures

G. resinaceum was initially grown on PDA medium in a petridish, and then transferred into the seed culture medium as a 5 mm of the agar plate culture. The precultures were carried out in 250 ml flasks containing 50 ml of MCM medium at 25°C for 4 days, using 4% (v/v) inoculation. All experiments were performed at least in triplicate to ensure reproducibility.

Batch and fed-batch fermentations in stirred-tank bioreactor

Fermentations were carried out using a 5-l stirred-tank bioreactor (Ko-BioTech Co., 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. Basically, the culture medium was the same as in flask culture. Samples were taken every 2 days for the analyses of mycelial dry weight, EPS and residual sugar concentration. For fed-batch cultures, various concentrations of glucose were supplemented in the medium when most of sugars were consumed at day 6.

Estimation of mycelial biomass and EPS concentration

Samples were collected 50 ml of medium at various intervals from shake flask and bioreactor and then centrifuged at 10,000 g for 20 min. The resulting supernatant was then filtered through a Whatman filter paper No. 2 (Whatman International Ltd., England). The resulting culture filtrate was mixed with four volumes of absolute ethanol, stirred vigorously and left overnight at 4°C. The EPS 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 EPSs were dialysed overnight against distilled water, followed by lyophilization and then the weight was estimated. After repeated washing of the mycelium with distilled water and drying at 70°C for 24 h to a constant weight, the dry weight of the mycelium was measured. The residual sugar content was determined by the phenol sulfuric acid method using glucose as the standard (Dubois *et al*., 1956).

SEC/MALLS analysis

The molecular weights of the EPS, were estimated by size exclusion chromatography (SEC) coupled with a multi angle laser light scattering (MALLS) system (DAWN DSP; Wyatt Technology, USA). The EPS samples were dissolved in a phosphate buffer (ionic strength = 0.1, pH 6.1) containing 0.04% ethylenediaminetetraacetic acid-disodium salt (Na_2 EDTA) and 0.01% sodium azide and filtered through 0.025 μm filter membranes (Millex HV type; Millipore Corp., USA) prior to injection into the SEC/MALLS

system (Hwang *et al*., 2003; Kim *et al*., 2003). 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 Corp., USA), an injection valve (Rheodyne Inc., USA) fitted with a 100 µl loop, the SEC columns (Shodex PROTEIN KW-803, 804; Showa Denko K.K., Japan) connected in series, and an RI detector (Water 410).

Chromatography was performed at room temperature. The flow rate was 0.5 ml/min. The injection volume and concentration were 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) was used according to the Wyatt Technology guide and data in the literature (Waytt, 1993), where estimated *dn/dc* was 0.14 ml/g. Calculation of molecular weights was performed using Astra 4.72 software (Wyatt Technology, USA).

Compositional analysis

The sugar composition was analyzed by gas chromatography (Varian STAR 3600CX, Varian Co. Model: Star 3600CX, Lexington, MA, USA) equipped with a flame-ionization detector on SP^{TM}-2380 capillary column (15 m × 0.25 mm, SUPELCO Co.,

Table 1. Effect of carbon sources on mycelial growth and exopolysaccharide (EPS) production in shake-flask cultures of *G. resinaceum* DG-6556[a]

Carbon sources (2%, w/v)	Mycelial dry weight (g/l)	EPS (g/l)
Xylose	1.69±0.18[b]	0.16±0.07
Lactose	1.91±0.20	0.57±0.12
Glucose	2.33±0.18	0.94±0.19
Sucrose	1.65±0.20	0.36±0.09
Mannitol	1.01±0.06	0.19±0.11
Maltose	1.67±0.20	0.77±0.10
Sorbose	0.93±0.17	0.50±0.03
Fructose	1.55±0.29	0.33±0.04
Starch	0.61±0.21	0.09±0.01

[a] Fermentations were carried out in shake flasks for 7 days at 25°C.
[b] Values are mean ±SD of triplicate experiments.

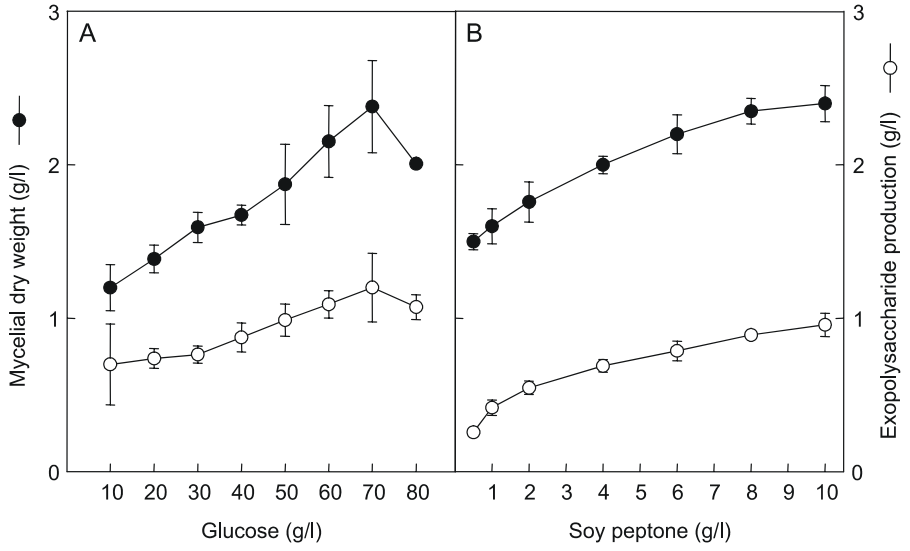

Fig. 1. Effect of initial carbon (A) and nitrogen concentration (B) on mycelial growth and exopolysaccharide (EPS) production in shake flask cultures of *G. resinaceum* DG-6556. The results were expressed as the average of triple determination with ±S.D.

Bellefonte, USA) with He as the carrier gas. For analysis of neutral sugars, the EPSs were hydrolyzed with 2 M trifluoroacetic acid (TFA) (3 h at 121°C). The resulting monosaccharides were quantified by gas liquid chromatography.

Results and Discussion

Optimization of nutrients for mycelial growth and EPS production

To find out the suitable carbon source for the EPS production and mycelial growth in *G. resinaceum*, nine important carbon sources were separately provided at 20 g/l. Among the carbon sources tested, glucose yielded the highest mycelial growth and EPS production (Table 1). Although the maximum final concentrations of mycelial biomass and EPS were achieved at an initial glucose level of 70 g/l, production yields of mycelial biomass and EPS based on consumed glucose were highest when initial 10 g/l of glucose was used (Fig. 1A).

To determine the best nitrogen source, individual nitrogen sources were supplemented into the medium in place of the yeast extract and meat peptone commonly used in MCM (Table 2). The highest mycelial biomass and EPS production were achieved in media containing soy peptone. As shown in Fig. 1, the optimum concentrations of carbon and nitrogen sources were 70 g/l and 8 g/l, respectively.

The effects of mineral sources on mycelial growth and EPS production were examined by supplementing various mineral sources at a concentration of 5 mM, in place of three mineral ions used in the basal medium. Enhanced production of mycelial biomass and EPS were observed when $MnCl_2$ was supplemented (Table 3).

Effect of initial pH and temperature

To investigate the effect of initial pH and temperature on mycelial growth and EPS production, the fungal cells were cultivated at various initial pHs and temperatures in 250 ml flasks containing 50 ml of optimized medium. The maximum mycelial growth and EPS production were obtained at an initial pH of 7.0 and 6.0, respectively (Fig. 2A). The optimum temperature for the culture mycelial growth was obtained at 28°C, whereas maximum EPS production was achieved at 31°C (Fig. 2B).

Effect of initial glucose concentration

Fig. 3 shows the typical time profiles of mycelial growth and EPS production of *G. resinaceum* using a

Table 2. Effects of nitrogen sources on mycelial growth and exopolysaccharide (EPS) production in shake-flask culture of *G. resinaceum* DG-6556[a]

Nitrogen sources (0.4%, w/v)	Mycelial dry weight (g/l)	EPS (g/l)
Sodium citrate	1.68±0.01[b]	0.42±0.03
Ammonium phosphate	1.75±0.01	0.15±0.06
Ammonium chloride	1.69±0.00	0.15±0.01
Ammonium citrate	1.84±0.01	0.57±0.18
Ammonium nitrate	1.65±0.01	0.22±0.02
Polypeptone	2.34±0.02	0.75±0.03
Casein peptone	2.30±0.01	0.68±0.02
Beaf peptone	1.81±0.01	0.55±0.22
Bacto peptone	1.77±0.01	0.61±0.05
Meat peptone	2.30±0.01	0.43±0.20
Soy peptone	2.94±0.04	1.05±0.21
Tryptone	2.22±0.01	0.64±0.01
Yeast extract	2.50±0.01	0.88±0.23
Corn steep powder	2.51±0.03	0.90±0.08
Corn steep liquor	2.57±0.02	1.07±0.15

[a] Fermentations were carried out in shake flasks for 7 days at 25°C.
[b] Values are mean ±SD of triplicate experiments.

Table 3. Effect of mineral sources on mycelial growth and exopolysaccharide (EPS) production in shake-flask cultures of *G. resinaceum* DG-6556[a]

Mineral sources (5 mM)	Mycelial dry weight (g/l)	EPS (g/l)
None	0.69±0.07[b]	0.40±0.11
$CaCl_2$	3.66±0.62	0.89±0.11
KNO_3	0.93±0.04	0.62±0.08
KH_2PO_4	0.96±0.31	0.55±0.05
K_2HPO_4	1.02±0.16	0.56±0.09
$MnCl_2$	1.67±0.53	1.58±0.19
$MgSO_4$	1.29±0.31	1.01±0.02

[a] Fermentations were carried out in shake flasks for 7 days at 25°C.
[b] Values are mean ±SD of triplicate experiments.

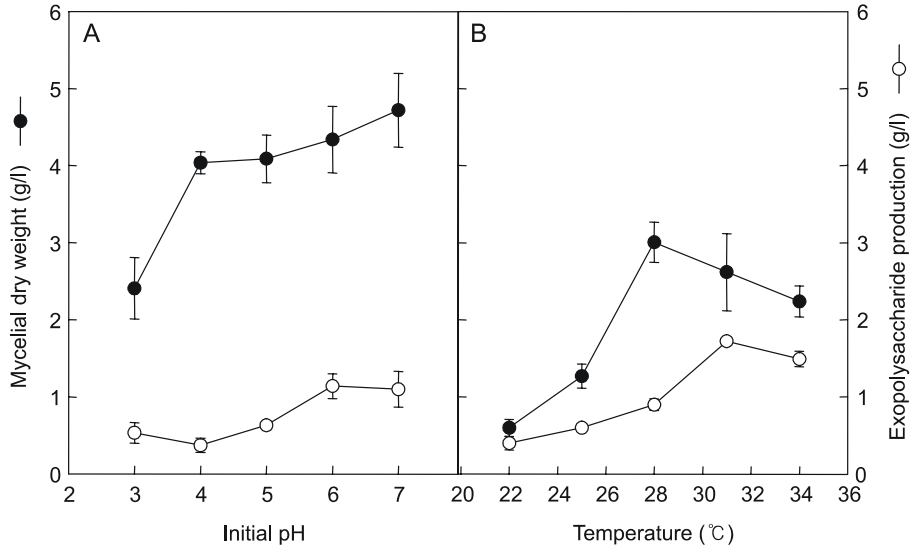

Fig. 2. Effect of initial pH (A) and temperature (B) on mycelial growth and exopolysaccharide (EPS) production in shake flask culture of *G. resinaceum* DG-6556. The results were expressed as the average of triple determination with ±S.D.

5-l stirred-tank bioreactor under three initial glucose concentrations (10, 35, and 70 g/l). The maximum concentrations of mycelial biomass at initial glucose levels of 10, 35, and 70 g/l were 3.5, 14.0, and 35.3 g/l on day 8, 14, and 14, respectively (Fig. 3B). The maximum EPS productions at initial glucose concentration of 10, 35, and 70 g/l were 1.4, 1.5, and 1.7 g/l on day 8, 10, and 12, respectively (Fig. 3C). The initial rates of total EPS concentrations (until day 4) were nearly the same irrespective of initial glucose concentration.

The greatest EPS concentrations were observed when an initial glucose concentration of 70 g/l was employed, until day 12. However, EPS production was higher with an initial glucose concentration of 35 g/l after day 12. This result implies that the optimum glucose concentration for EPS production would be achieved at a glucose concentration between 35 and 70 g/l. The overall time profiles of top and bottom fractions of EPS were similar. However, Top-EPS was not detected after day 8 for those with an initial glucose concentration of 10 g/l. This result is probably due to the fungus utilizing Top-EPS as a carbon source once the glucose was depleted in the culture medium. The EPS yield versus glucose used, denoted as value 'Y', decreased with an increase of initial glucose concentration, where their values were 0.092, 0.066, and 0.061 *g*/g at an initial glucose level of 10, 35, and 70 g/l, respectively (Table 4).

In submerged cultures of bioactive polysaccharide-producing higher fungi, such as Basidiomycetes and Ascomycetes, nutritional and environmental conditions are particularly important, as they significantly affect the production of polysaccharides and their quality

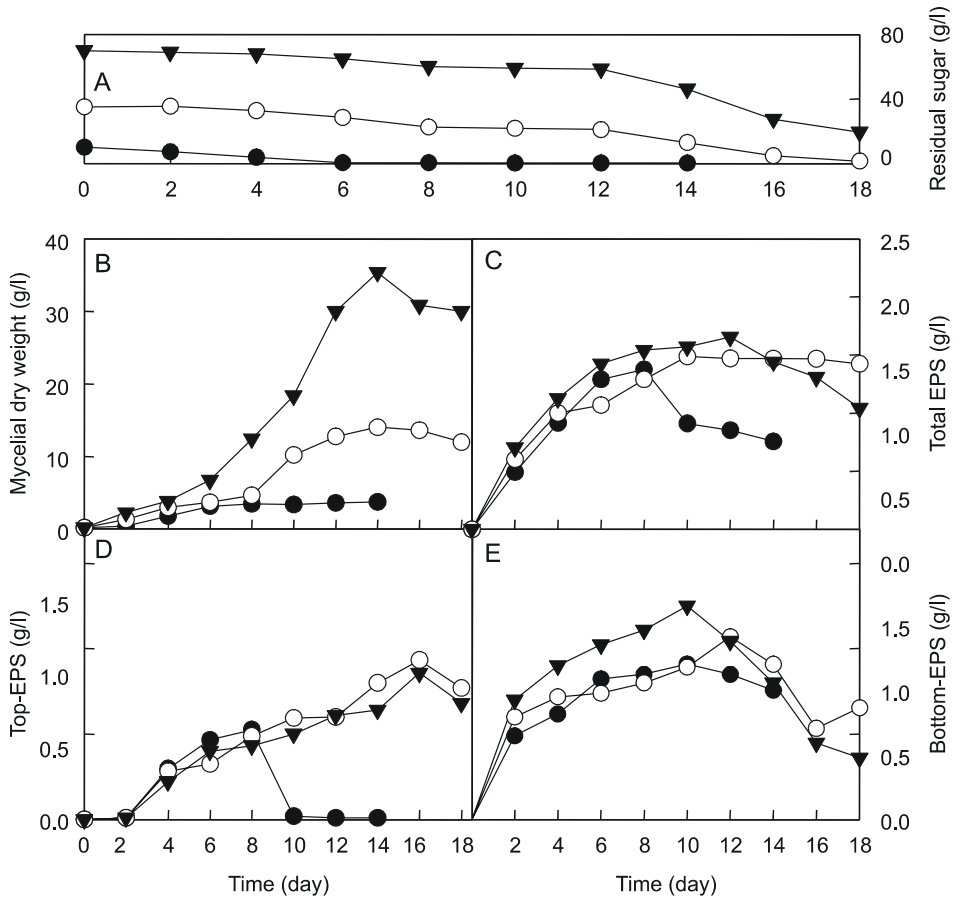

Fig. 3. Time profiles of the mycelial growth and exopolysaccharide (EPS) production at different initial glucose concentrations in submerged culture of *G. resinaceum* DG-6556 using 5-l stirred-tank bioreators. Top- and Bottom-EPS indicate top fraction of EPS and bottom fraction of EPS in ethanol precipitate of culture filtrate, respectively. Initial glucose concentration: (●) 10 g/l; (○) 35 g/l; (▼) 70 g/l.

Table 4. Fermentation kinetics in batch and fed-batch fermentations by submerged culture of *G. resinaceum* DG-6556 for the production of exopolysaccharides (EPS)

Kinetic parameter	Batch			Fed-batch		
Substrate, S (g/l)	10	35	70	10+10	10+25	10+50
Maximum biomass, X (g/l)	3.46 (8 days)	14.02 (14 days)	35.34 (14 days)	11.54 (12 days)	19.72 (14 days)	42.22 (14 days)
Maximum EPS, P (g/l)	1.38 (8 days)	1.49 (10 days)	1.65 (12 days)	1.95 (8 days)	3.04 (12 days)	4.59 (12 days)
Yield of EPS on cell mass[a], $P_{P/X}$ (g/g)	0.048	0.013	0.003	0.093	0.014	0.013
Yield of EPS on substrate[a], $Y_{P/S}$ (g/g)	0.092	0.066	0.061	0.211	0.189	0.180

[a] Yields were estimated from initial rates of EPS formation during the first 6 days.

(Jonathan and Fasidi, 2001; De Baets *et al*., 2002; Kim *et al*., 2003). Many investigators have studied the effects of carbon sources and initial sugar concentration in order to enhance the final cell density and production of useful metabolites (Bae *et al*., 2000; Park *et al*., 2001; Bhargava *et al*., 2002). By using a low initial glucose concentration and intermittent feeding during fermentation, the inhibitory effect of a relatively higher initial glucose concentration on the polysaccharides production was avoided and the process efficiency was greatly enhanced (Bhargava *et al*., 2002).

Fed-batch culture

On the basis of batch fermentation results, fed-batch

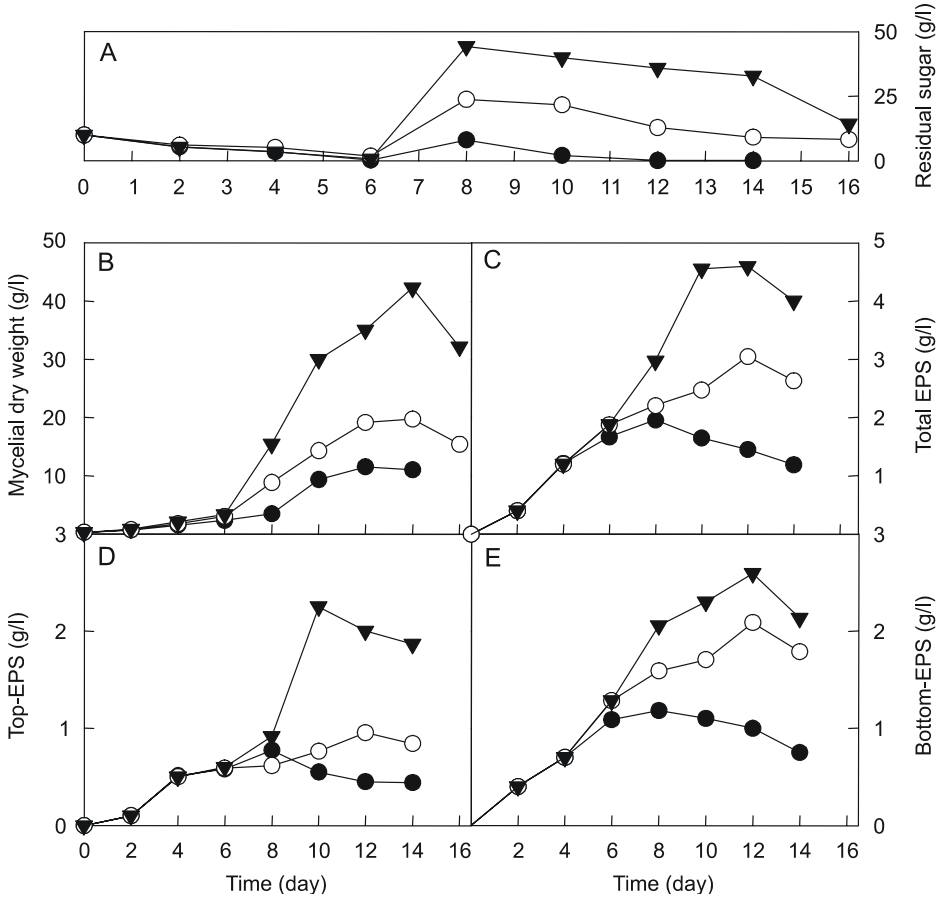

Fig. 4. Time profile of the mycelial growth and exopolysaccharide (EPS) production in fed-batch culture of *G. resinaceum* DG-6556 by feeding different amount of glucose using 5-l stirred-tank bioreators. Top- and Bottom-EPS indicate top fraction of EPS and bottom fraction of ethanol precipitate of culture filtrate, respectively. The vertical arrows indicate time of glucose feeding (day 6). Feeding glucose concentration: (●) 10 g/l, (○) 25 g/l, (▼) 50 g/l.

Table 5. Molecular weights of top and bottom fractions of EPS (Fr-I~IV) obtained from culture filtrate during ethanol precipitation with the SEC/MALLS system

EPS[a]	*M*w x 10^5 g/mol (error %)			
	Fr-I	Fr-II	Fr-III	Fr-IV
Top	39.720 (2.3)	1.210 (0.3)	0.458 (1.0)	-
Bottom	52.570 (2.7)	1.548 (0.9)	0.671 (1.0)	0.530 (1.1)

[a] Samples were taken at day 8 (also see Fig. 5).

cultures were performed with three different feeding strategies. Starting with an initial 10 g/l of glucose, three different glucose concentrations (*e.g.* 10, 25, and 50 g/l) were fed into the culture medium at day 6 when most of initial glucose was depleted (Fig. 4A). As shown in Fig. 4B, maximum concentrations of mycelial biomass at feeding glucose levels of 10, 25, and 50 g/l were 11.5, 19.7, and 42.2 g/l on day 12, 14, and 14, respectively. Correspondingly, maximum EPS concentrations were 1.9, 3.0, and 4.6 g/l on day 8, 12, and 12, respectively. Compared with batch culture results at an initial glucose concentration of 35 g/l, it is notable that the maximum concentration of mycelial biomass (from 14.0 g/l to 19.7 g/l) and EPS (from 1.5 g/l to 3.0 g/l) achieved in fed-batch culture by feeding 25 g/l of glucose (total amount of glucose fed = 35 g/l), were significantly enhanced. This yield is an underestimation due to the dilution effect of the glucose feeding.

The strategy of fed-batch fermentation is to add

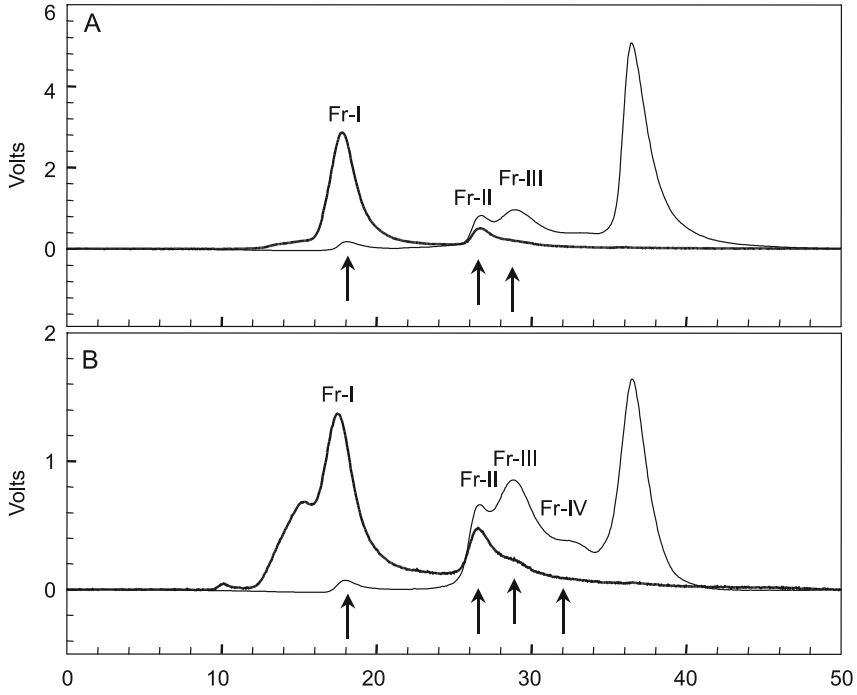

Fig. 5. Elution profiles of EPS for the determination of molecular weights in the SEC/MALLS system. For detailed analysis conditions, see Materials and methods section. (A) Elution profiles of top fractions of ethanol precipitate of culture filtrate, (B) elution profiles of bottom fractions of culture filtrate during ethanol precipitation. Thick lines indicate the elution profiles from the MALLS detector, indicating each molecular weight of EPS, while thin lines indicate the elution profiles from the refractive index detector, showing the existence of different EPS (see vertical arrows). The high peaks appearing around elution volumes of 36 ml are a result of baseline noise by the buffer solution.

one or more of the nutrients during fermentation. This is based on the possibility that the high concentrations required for high final cell growth and product yields might inhibit growth, if added in total at the start of fermentation. Potentially, growth and product formation could be extended for longer periods compared to normal batch fermentation.

There are several reports describing submerged culture of mushrooms using the fed-batch method (Jonathan and Fasidi, 2001; Tang and Zhong, 2002; Wagner *et al*., 2004). Tang and Zhong (2002) developed an efficient process for simultaneously producing bioactive ganoderic acid and polysaccharide by fed-batch fermentation of *G. lucidum*. The authors demonstrated that fed-batch cultures were very useful for highly efficient accumulations of biomass, polysaccharides, and ganoderic acid. De Baets *et al*. (2002) reported that EPS production was increased by 1.6-fold using single fed-batch cultures and by 2.2-fold using cyclic fed-batch cultures of the mushroom *Tremella mesenterica*, compared to the reference batch fermentation.

In the present study, it was found that mycelial growth was less efficient at higher sugar concentrations, possibly due to increased maintenance requirements of the fungus. For instance, the yield coefficient of EPS on consumed glucose was significantly decreased from 0.092 at 10 g/l of glucose to 0.061 at 70 g/l of glucose in batch fermentation (Table 4). From this result, a fed-batch culture strategy was adopted and proved to be efficient for enhanced production of mycelial biomass and EPS. The enhanced EPS and mycelial biomass production by fed-batch cultures in this study were probably due to a decrease in the osmotic pressure of the culture medium or by prevention of a catabolite regulating effect exerted by the rapidly degradable carbon source, glucose (Jonathan and Fasidi, 2001). When the yields of mycelial biomass and EPS in fed-batch fermentation were compared with those of batch fermentation of the same glucose concentration (35 g/l), 5.72 g/l of extra mycelial biomass and 1.55 g/l of extra EPS were produced by fed-batch cultures.

Molecular features of EPS

The top and bottom fractions of EPS solution were individually forwarded to the SEC system. Their molecular weights were also determined separately by the MALLS system. Fig. 5 shows the elution profiles of the top and bottom fractions of EPS. Three and four different EPS were eluted from top and bottom fractions of culture filtrates, respectively. The

molecular weights of Top-EPS ranged from 45,800 to 3,972,000 g/mole, while those of Bottom-EPS ranged from 53,000 to 5,257,000 g/mole (Table 5). Lower molecular weight EPSs were abundant in both top and bottom fractions.

In this study, we obtained a total of seven different EPSs from culture filtrate during ethanol precipitation with the SEC/MALLS system. The results of compositional analysis revealed that EPS consisted of 85~92% glucose with small amounts of mannose, fucose, galactose, and xylose. However, the biological activities of each EPS were not studied and deserve further investigation. In conclusion, the fed-batch culture was proved to be a useful process for enhancing the production of both mycelial biomass and EPS in *G. resinaceum*. It may be worth attempting the fed-batch culture method with other mushroom fermentation processes for enhancing the production of mushroom polysaccharides, particularly those with industrial potential.

Acknowledgement

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