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Factors Influencing the Production of Water-soluble Endopolysaccharides and Exopolysaccharides from *Lentinus lepideus* and their Effects on Immune Cytokine Production

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An efficient method to produce water-soluble polysaccharides from *Lentinus lepideus* is described. The productivity of both endopolysaccharides (PPS) and exopolysaccharides (EPS) was compared under various culture conditions. The effect of treating their own PPS and EPS on immune cytokine production was also studied in relation to culture factors. High yield production of EPS required a moderate culture temperature (25°C) as well as long culture period (16–20 days). In contrast, PPS production required a high culture temperature (30°C) and short culture period (8 days). Most of the carbon sources did not affect polysaccharides and mycelial production except for sucrose. Immune cytokine levels in the EPS treatment varied among carbon sources or culture periods. PPS did not appear to affect much on the production of cytokines, regardless of the culturing factors, except for the culture period. These results suggest that the optimal culture conditions for *L. lepideus* vary according to culture purposes, and different culture conditions should be used for different targets including mycelial biomass, EPS, and PPS. Whereas the immunomodulating activitiy of EPS appeared to be affected by culture conditions in *L. lepideus*, that of PPS did not.

Keywords: *Lentinus lepideus*, mycelial culture, exopolysaccharide, endopolysaccharide, immune cytokine

Lentinus lepideus, a *Basidiomycete* fungus, belongs to the family Polyporaceae. Its strong anticancer activities have attracted much interest. Specifically, a water-soluble glucan from *L. lepideus* is known to induce B cell proliferation in the mouse system [11]. A water-soluble extract, PG101, from *L. lepideus* has also been shown to activate cellular transcription factor NF-B and control the expression of various cytokines including TNF-α, IL-1, IL-10, IL-12, IL-18, and GM-colony-stimulating factor (CSF) in human peripheral blood mononuclear cells (PBMCs) [10, 13]. Such cytokines as IL-1 and GM-CSF have been reported for their therapeutic effects on hematopoiesis and immune cells by stimulating bone marrow regeneration [9]. The data suggest that PG101 could be a potential biological response modifier (BRM) and thus could be used as an immune enhancer in immunocompromised and immunosuppressed individuals. Previously, we demonstrated that the biological substance of water extracts from *L. lepideus* was polysaccharides, as in most other mushrooms.

As the production of polysaccharides is more efficient from mycelia than from fruit bodies, the influence of culture conditions on mycelial production has drawn much attention [5, 19, 28, 32]. Most of the fungal polysaccharides mediating biological activities are endopolysaccharides (PPS) [16, 29] or exopolysaccharides (EPS) [1, 3, 4, 34]. However, despite their potential usefulness, *L. lepideus* has not been extensively tested for their potential for polysaccharide production in submerged culture. Several investigators have pointed out that both culture medium and environmental conditions affect most the production and the physicochemical properties of EPS [8, 18, 25, 28]. The sugar composition of EPS depends on the medium sources [18], whereas its molecular weight varies with aeration conditions [33] or culture period [21]. Biological activities of EPS are known to be affected by its molecular weight [2, 26]. The physicochemical properties of polysaccharide are also reported to be strongly influenced by several factors, including sugar composition and the degree of polymerization [12]. Therefore, it is very important to optimize culture conditions for the production of a specific type of polysaccharides from submerged culture.

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In addition to polysaccharides extracted from fruiting bodies, PPS from submerged mycelial culture have also drawn some attention [16]. However, to our knowledge, the influence of culture conditions on physicochemical properties and their immune cytokine production capacities of PPS have not been reported. The capacity of immune cytokine production could be estimated by determining the TNF-α level, since it is a major immune cytokine that modulates variable cytokine levels and activates immune cells [6].

In the present study, we tested various culturing factors that might influence the production of mycelial biomass, PPS, and EPS in submerged culture of *L. lepideus*, analyzed the chemical characteristics of the polysaccharides, and compared the effects of culturing factors on immune cytokine production by treating their own PPS and EPS.

MATERIALS AND METHODS

Microorganism and Inoculum Preparation

The *L. lepideus* strain KFRI 646 was obtained from the Korea Forest Research Institute. The stock culture was maintained on potato dextrose agar slants. The seed cultures were grown in 500-ml flasks containing 200 ml of Fungal Growth Medium (FGM) containing, per liter, 20 g of glucose, 3 g of yeast extract, 1 g of glutamic acid, 0.1 g of thiamine, 0.1 g of KH_2PO_4, 0.05 g of $MgSO_4$, 5 ml of 0.1 M $FeCl_3$, and 5 ml of 0.1 M $MnSO_4$ at 25°C on a shaking incubator at 100 rpm for 10 days. The cultures were harvested from the culture, homogenized at 13,000 rpm for 8 sec in a homogenizer (Ingenieurbüro CAT. X1030D, M. Zipperer GmbH, Germany), and used as inoculum.

Culture Conditions

Flask culture experiments were performed in 500-ml flasks containing 200 ml of FGM, by inoculating seed culture to 4% (v/v) of the total volume for 10 days at a temperature of 25°C. The initial pH of the media was adjusted to 4.2 with HCl and KOH. Fermentations were carried out both in a 5-l balloon-type air bubble bioreactor (BTBB) [20] and in a 5-l stirrer-type bioreactor (STBB; Hankook Fermentation Co., Korea) for 10 days at a temperature of 25°C, with an air flow rate of either 0.1 vvm (BTBB) or 0.1 vvm with an agitation of 80 rpm (STBB). For fermentation, FGM was used as basal medium with a 4-l working volume.

Estimation of Mycelial Biomass and Production of EPS and PPS

Samples collected from various treatments were centrifuged at 3,700 ×*g* for 20 min to separate mycelia from culture solution. The mycelia were washed with distilled water, lyophilized, and weighed for their dry weight. We modified the method of Kim *et al.* [14] for the production of EPS and PPS. To extract EPS, cultured solution was mixed with four volumes of absolute ethanol, stirred vigorously, and then left overnight at 4°C for precipitation. The precipitated EPS was centrifuged at 3,700 ×*g* for 20 min, redissolved with distilled water and centrifuged again. The EPS was weighed after lyophilizing the supernatant. To extract PPS, the dry mycelia were washed three times with 80% ethanol, submerged in distilled water, autoclaved at 120°C (1.5 atm) for 1 h, and centrifuged. The filtrate was mixed with four volumes of absolute ethanol, stirred vigorously, and then left overnight at 4°C for precipitation. The precipitate was centrifuged at 3,700 ×*g* for 20 min, redissolved with distilled water, and centrifuged again. The supernatant was dialyzed and lyophilized before weighing the PPS.

Analysis of Carbohydrate and Protein

Glucose content in the media was analyzed HPLC by on a Prevail Carbohydrate ES column (250×4.6 mm, 5 μm; Alltech Associates, IL, U.S.A.), using isocratic elution with acetonitrile/H_2O (72/28, v/v) and an ELSD detector (ELSD 2000; Alltech Associates, IL, U.S.A.). Total carbohydrate content in polysaccharides was determined by the phenol-sulfuric acid method [7] using glucose as a standard. Sugar composition of the polysaccharide was analyzed by high-performance anion-exchange chromatography (HPAEC) on a CarboPac PA10 anion-exchange column (250×4 mm; Dionex Corporation, CA, U.S.A.), by isocratic elution with 20 mM NaOH for 30 min using a pulsed amperometric detector (PAD; Dionex Corporation, CA, U.S.A.) after acid hydrolysis with 72% (w/w) H_2SO_4 at 30°C for 45 min, followed by 4% (w/w) H_2SO_4 for 1 h at 120°C (1.5 atm) in an autoclave [27]. Total protein was determined by the Bradford method with bovine serum albumin as a standard.

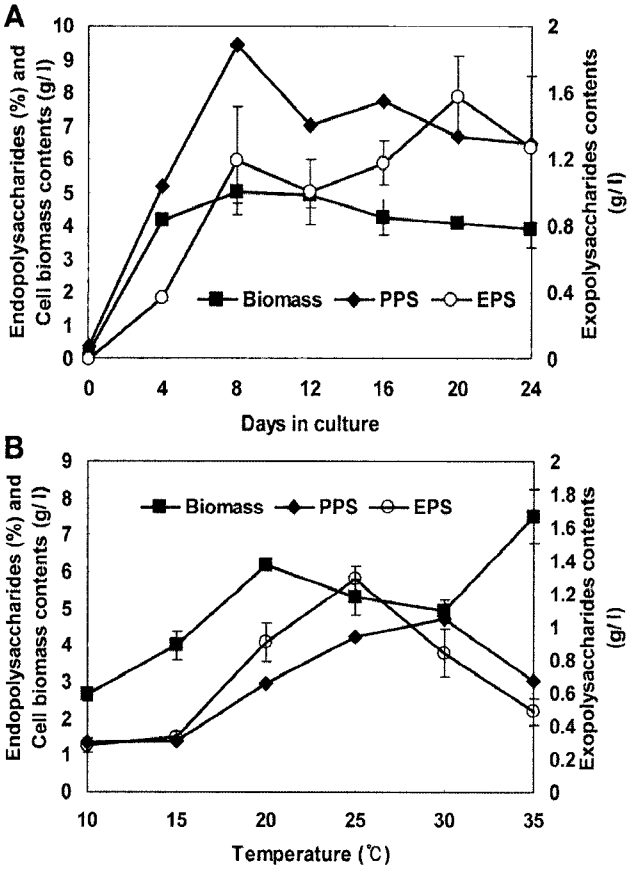

Fig. 1. Effects of culture periods (**A**) and temperature (**B**) on the production of mycelial biomass (g-dw/l), water-soluble endopolysaccharides (PPS; %, w/w of cell), and water-soluble exopolysaccharides (EPS; g/l) from *Lentinus lepideus* in shake-flask culture.

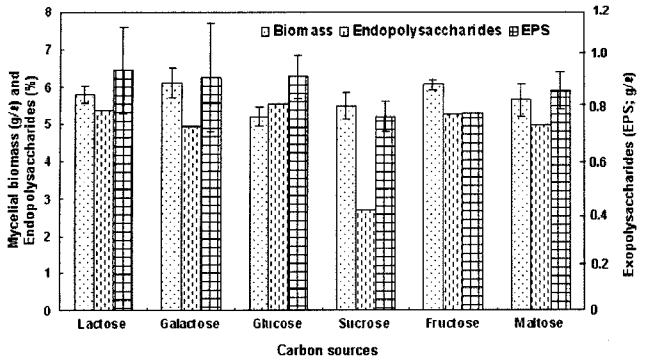

Fig. 2. Effects of carbon sources on the production of mycelial biomass (g-dw/l), water-soluble endopolysaccharides (%, w/w of cell), and water-soluble exopolysaccharides (g/l) from *Lentinus lepideus* in shake-flask cultures at 25°C for 10 days.

Molecular Mass Determination

The molecular masses of the EPS were estimated on the basis of the calibration curve obtained by HPLC (Spectra System, TSP, CA, U.S.A.) with a Shodex Sugar KB-805 column (0.8×30 cm; Showa Denko K.K., Tokyo, Japan) using distilled water as the mobile phase (column temperature 50°C; flow rate 0.8 ml/min) [17]. The column was standardized with dextrans of diverse molecular mass. The detector used was RI (Waters 410 D.R.; Waters, MA, U.S.A.).

Measurement of TNF-α and IL-10 Levels

Human peripheral blood mononuclear cells (PBMCs) were used to produce tumor necrosis factor (TNF-α) and interleukin-10 (IL-10) by treating PPS or EPS. PBMCs were prepared by the method of Jin *et al.* [10]. The cells were incubated with PPS or EPS at the concentration of 100 μg/ml at 37°C under an atmosphere containing 5% CO_2 for 24 h. Levels of cytokines (TNF-α and IL-10) were measured using commercially available ELISA kits (Endogen, Woburn, MA, U.S.A.) according to the manufacturer's instruction. The supernatants from the cell cultures were tested for the cytokine contents.

Results and Discussion

Influences of Culture Conditions on the Production of Mycelial Biomass and Polysaccharides

To determine the optimal culture period for the production of PPS and EPS, *L. lepideus* was cultured in flasks with agitation for 4 to 24 days. The cell growth kinetic and production profiles of PPS and EPS are shown in Fig. 1A. Stationary phase was reached in 8 days and death phase was after 12 days in culture. Cell biomass (g/l) and PPS content in the cell (%, w/w of cell) also reached a peak after 8 days in culture. The highest EPS production was obtained from culture broth at the 20th day. Thus, both EPS and PPS require different optimal culture periods for production.

In the subsequent experiment, we fixed the culture period to 10 days, as both the production of mycelia and

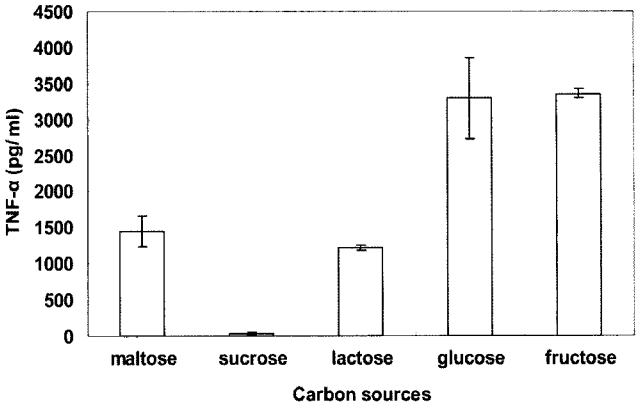

Fig. 3. Effect of water-soluble exopolysaccharides (EPS) on TNF-α secretion by treating their own obtained from submerged culture of *Lentinus lepideus* under various carbon sources.
The mycelia were cultured in shake flasks at 25°C for 10 days. PBMCs were treated with 100 μg/ml EPS, and the level of TNF-α was measured by ELISA kit after 24 h.

EPS were more stable in 10-day culture than in 8-day culture under the same conditions. The EPS obtained from 12-day culture appeared to be low in their water solubility.

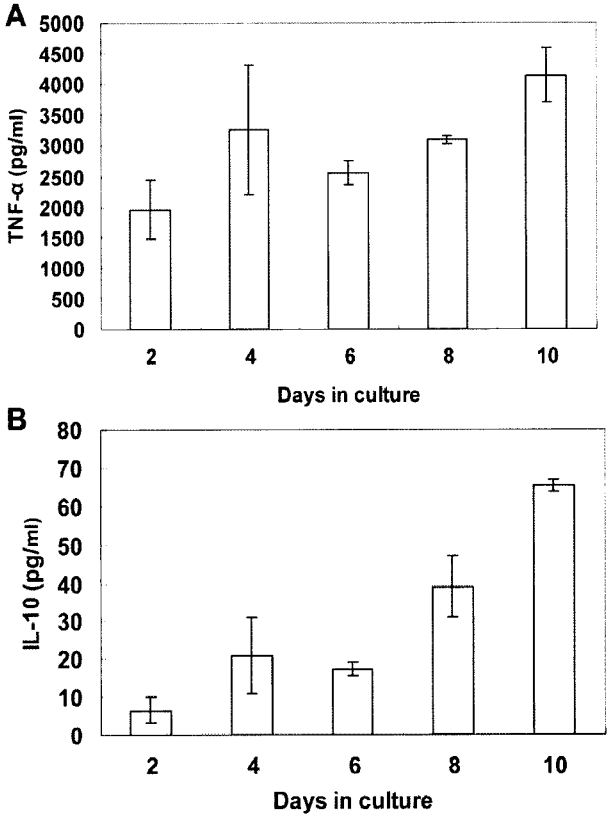

Fig. 4. Effect of water-soluble exopolysaccharides (EPS) on TNF-α and IL-10 secretion by treating their own obtained from submerged culture of *Lentinus lepideus* under various culture periods.
The mycelia were cultured in a balloon-type air bubble bioreactor at 25°C. PBMCs were treated with 100 μg/ml EPS, and the level of TNF-α (**A**) and IL-10 (**B**) were measured by ELISA kit after 24 h.

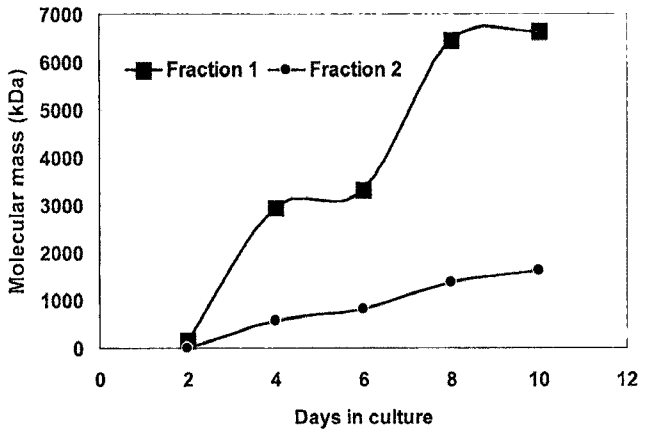

Fig. 5. The time-course change in molecular mass of water-soluble exopolysaccharides during the submerged culture of *Lentinus lepideus*.
The mycelia were cultured in a balloon-type air bubble bioreactor.

To investigate the effect of temperature on the production of cell biomass, PPS and EPS, *L. lepideus* were exposed to various temperature conditions for 10 days (Fig. 1B). Optimal culture temperatures for the highest cell biomass, PPS content in the cell, and EPS turned out to be 35, 30, and 25°C, respectively. The results suggest that the mycelia accumulate polysaccharides within the cells at high temperature. However, PPS productions per liter of media (g/l) were almost the same at culture temperature from 25 (22.5 g/l) or 30°C (23.5 g/l). The yield of EPS increased as culture temperature increased from 15 to 25°C, and decreased from 25°C to higher. Similar results for EPS production have been reported from submerged culture of *Agrocybe cylindracea* [14] and *Grifola frondosa* [17]. The results indicate that different optimal temperatures are needed to produce PPS and EPS from *L. lepideus*.

In most cases, the mycelia tended to grow on a wide range of carbon sources [35]. To find out the effects of different carbon sources on the production of cell biomass, EPS, and PPS, five carbon sources were compared. Cell biomass, EPS, and PPS from submerged culture of *L. lepideus* did not show any significant difference according to carbon sources in the media, except for sucrose (Fig. 2). The PPS content obtained from sucrose-containing medium was a half of those produced by the media containing other carbon sources. However *L. lepideus* did not appear to require specific carbon sources for the production of cell biomass, EPS, or PPS from submerged culture.

Table 2. Molecular masseds of water-soluble exopolysaccharides producing from *Lentinus lepideus* mycelia at culture condition of various carbon sources. The mycelia were cultured in shake flasks at 25°C for 10 days.

Carbon sources	Molecular mass (kDa)	
	Fraction 1	Fraction 2
Glucose	4,600	2,320
Maltose	3,160	-
Fructose	3,340	-
Sucrose	2,280	-
Lactose	2,240	1,350

Effect of Culture Conditions on Properties of EPS and their Immune Cytokine Production

To compare the effects of culture conditions on immune cytokine production, human peripheral blood mononuclear cells (PBMCs) were used. Levels of both TNF-α and IL-10 in the cell culture supernatant were measured using a commercial ELISA kit. PBMCs produced various cytokines, including TNF-α, IL-1, IL-10, IL-12, IL-18, and GM-colony-stimulating factor by treating with *L. lepideus* extracts [10]. TNF-α is a major immune cytokine that modulates variable cytokine levels and activates immune cells [6].

Fig. 3 shows the TNF-α production by treating EPS obtained from *L. lepideus* cultured in the presence of various carbon sources. EPS fed with glucose and fructose induced more TNF-α than did those provided with other carbon sources. The biological activity of EPS obtained from *L. lepideus* was affected by their carbon sources used in the media. As shown in Fig. 4, EPS stimulated TNF-α or IL-10 production in a time-dependant manner. The longer the cultivation period, the more TNF-α or IL-10 was secreted. The biological activity of EPS also seemed to be affected by the cultivation periods of the mycelium.

Table 1. Contents of proteins and carbohydrates, and composition of the carbohydrates in water-soluble exopolysaccharides produced from *Lentinus lepideus* mycelium at culture condition of various carbon sources. The mycelia were cultured in flasks at 25°C for 10 days.

Carbon sources	Content (%, w/w)		Sugar component (%, w/w)				
	Protein	Carbohydrate	Fucose	Glucose	Galactose	Mannose	Xylose
Fructose	1.21	80.80	0.25	87.89	2.76	0.17	8.93
Glucose	1.34	77.43	0.25	89.35	1.65	0.16	8.59
Galactose	1.05	85.15	0.40	84.78	4.24	0.16	10.42
Sucrose	1.44	90.41	0.20	90.70	2.05	0.15	6.88
Lactose	0.97	80.46	0.37	87.59	2.62	0.16	9.26
Maltose	1.28	86.71	0.40	84.97	3.76	0.19	10.68

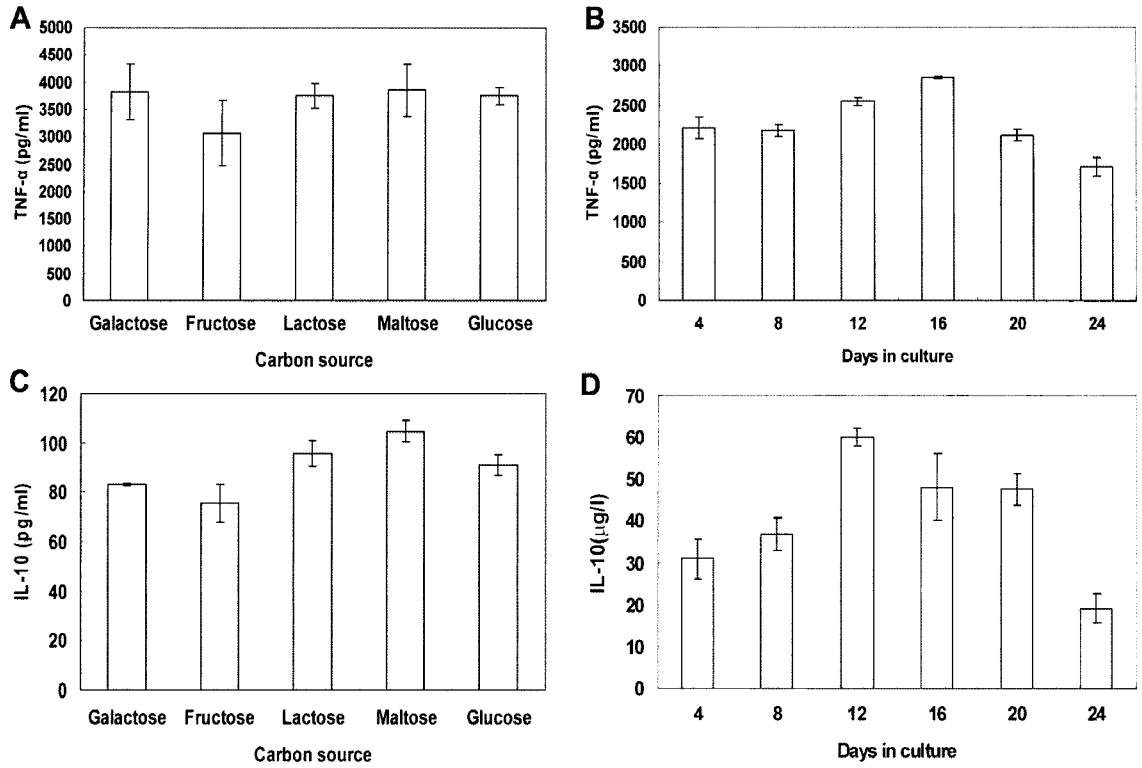

Fig. 6. Effect of water-soluble endopolysaccharides (PPS) on TNF-α and IL-10 secretion by treating their own obtained from submerged culture of *Lentinus lepideus* under various carbon sources (**A**, **C**) and culture periods (**B**, **D**).
The mycelia were cultured in shake flasks at 25°C for 10 days. PBMCs were treated with 100 μg/ml PPS, and the levels of TNF-α and IL-10 were measured by ELISA kit after 24 h.

In order to find out the effects of chemical properties of EPS on the immune cytokine production, their molecular masses (Fig. 5) and sugar compositions were determined (Table 1). EPS from submerged culture media are commonly known to be separated into two main fractions [17, 33]. Likewise, our EPS from *L. lepideus* were separated into two main fractions (Table 2). Although sugar compositions of EPS obtained in the presence of the various carbon sources did not vary much, their molecular masses clearly showed difference by carbon sources used (Table 2) or culturing periods of *L. lepideus*. As shown in Fig. 5, the molecular mass of EPS changed with culture period. The longer it was cultured, the higher the molecular mass of the biopolymer. In the growth kinetics of *L. lepideus*, the culture entered the growth phase after 4 days, reached the stationary phase after 8 days, and finally settled in the death phase after 10 days in submerged culture in the bioreactor. EPS obtained after the stagnant phase had the highest molecular mass and also induced higher amount of immune cytokines. In the submerged culture of *L. lepideus*, both glucose and fructose used as carbon sources produced a higher molecular size of EPS. The EPS produced in the presence of either glucose or fructose also induced a higher amount of immune cytokines than did any other carbon sources used.

However, the cytokine level increased as the molecular mass of EPS became higher. Biological activity of EPS

Table 3. Contents of proteins and carbohydrates, and composition of the carbohydrates in water-soluble endopolysaccharides produced from *Lentinus lepideus* mycelia at culture condition of various carbon sources. The mycelia were cultured in flasks at 25°C for 10 days.

Carbon sources	Content (%, w/w)		Sugar component (%, w/w)				
	Protein	Carbohydrate	Fucose	Glucose	Galactose	Mannose	Xylose
Fructose	1.66	96.71	0.20	87.74	5.45	0.21	6.40
Glucose	1.97	97.00	0.32	89.30	5.06	0.43	4.89
Galactose	1.82	94.68	0.22	88.96	4.97	0.17	5.69
Sucrose	1.99	98.00	0.15	89.24	4.27	0.2	6.12
Lactose	0.16	94.24	0.29	87.54	5.29	0.11	6.77
Maltose	1.39	93.78	0.24	89.26	3.59	0.36	6.56

Table 4. Contents of proteins and carbohydrates, and composition of the carbohydrates in water-soluble endopolysaccharides produced from *Lentinus lepideus* mycelia at culture condition of various carbon sources. The mycelia were cultured in shake flasks at 25°C for 10 days.

Days in culture	Content (%, w/w)		Sugar component (%, w/w)				
	Protein	Carbohydrate	Fucose	Glucose	Galactose	Mannose	Xylose
4	2.71	84.75	0.10	88.79	6.09	0.10	4.92
8	1.76	88.55	0.14	88.72	4.53	0.10	6.50
12	1.97	97.00	0.32	89.30	5.06	0.43	4.89
16	2.35	90.99	0.52	84.00	6.45	0.91	8.12
20	2.17	91.32	0.61	77.21	9.90	1.31	10.97
24	1.25	97.09	0.50	80.03	8.33	1.25	9.89

was reported to be affected by its molecular mass [2, 26]. A high molecular mass was more effective for glucan's antitumor activity than a low molecular mass [22, 23]. Thus, it is necessary to optimize the culture period to obtain the right molecular mass of the target product.

Effect of Culture Conditions on Properties of PPS and their Immune Cytokine Production

Polysaccharides extracted from some basidiomycete fungi are well known to act as biological response modifiers (BRM) [30, 31]. However, the physicochemical properties of the polysaccharides could change by culturing conditions [12]. In the present study, PPS obtained from *L. lepideus* mycelia under variable culture conditions were compared for their capacities to produce immune cytokines from PBMCs and their chemical properties. Fig. 6 shows the immune cytokine activities of PPS obtained from *L. lepideus* with various carbon sources for different culturing periods. Whereas immune cytokine activities of PPS obtained under various carbon sources did not vary much (Table 3), those of PPS obtained from different culturing period treatments showed visible differences (Table 4).

PPS extracted from 12- to 16-day culture induced a higher amount of immune cytokine than did those from the remaining treatments. As for the chemical properties of PPS obtained under the variable carbon sources, both protein and carbohydrate contents and sugar composition did not seem to differ much among different carbon sources treated. However, the sugar composition of PPS obtained under different culturing periods tended to decrease their glucose content as the culturing days proceeded. Therefore, the chemical properties of PPS appear to undergo some modification with culture age.

Effect of Bioreactor Types on Polysaccharides Production and Production of Immune Cytokine by Treating their Polysaccharides

To investigate the influence of different bioreactor types on the production of mycelial biomass and polysaccharides from *L. lepideus*, both balloon-type and stirrer-type air bubble bioreactors were compared. The polysaccharides from those bioreactors were also studied with respect to their immune cytokine production activities from PBMCs. The balloon-type air bubble bioreactor (BTBB; 5.29 g/l) produced more mycelial biomass than did the stirrer-type (STBB; 2.93 g/l) (Table 5). Mycelia grew as pellet-like form in the BTBB but showed feather-like form in the STBB. Shearing stress, even though the rotating speed was 80 rpm, had a detrimental effect to the formation of mycelial pellets in the STBB. Low aeration rate (0.5 vvm) as well as low agitation speed (100 rpm) are commonly known to be beneficial for mycelial growth in submerged culture of mushroom [15]. EPS production was higher in BTBB (0.61 g/) than in STBB (0.41 g/). PPS content in the cell was also higher in BTBB (0.78%) than in STBB (0.69%). Taken these together, BTBB appears to be more suitable than STBB to produce mycelia and polysaccharides from *L. lepideus*. Both mycelial biomass and polysaccharides production from fermentation

Table 5. Comparison of growth characteristics of *Lentinus lepideus* cultured at balloon and stirrer types of air bubble bioreactors.

Type of bioreactor	Cell biomass (g-dw/)[a]	Days of culture	Overall cell yield, Yx/s[b]	Overall growth rate Rx (g/d/)[c]	Yield of PPS[d] (g/)	PPS content in cell (%)	Yield of EPS[e] (g/)
Balloon	5.29	10	0.37	0.53	0.41	0.72	0.61
Stirrer	2.93	10	0.21	0.29	0.23	0.68	0.41

[a]Mycelial dry weight after cultivation of 10 days.
[b]The initial glucose concentration was 20 g/l. The value of Yx/s was defined as the cell mass obtained per gram of glucose consumed.
[c]The value of Rx was defined as the cell concentration at the end of the culture divided by the total cultivation time.
[d]Water-soluble endopolysaccharides.
[e]Water-soluble exopolysaccharides.

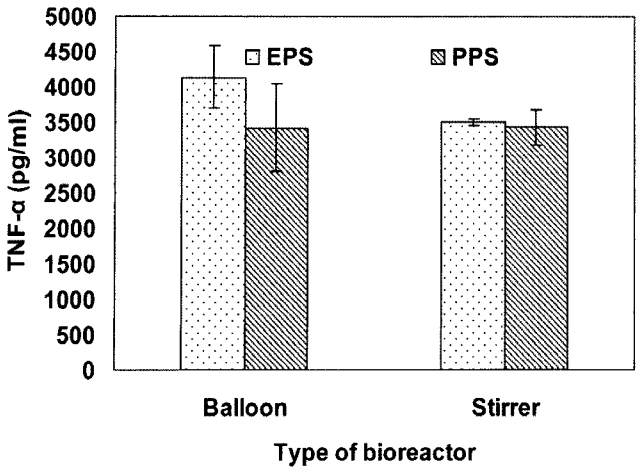

Fig. 7. TNF-α secretion by treating water-soluble endopolysaccharides (PPS) and water-soluble exopolysaccharides (EPS) obtained from submerged culture of *Lentinus lepideus* in balloon and stirrer types of air bubble bioreactors.
PBMCs were treated with 100 μg/ml polysaccharides and the level of TNF-α was measured by ELISA kit after 24 h.

of mushrooms are principally affected by types of bioreactors, aeration, or agitation rate [29, 33]. Thus, a higher amount of polysaccharides could be produced from *L. lepideus* when the fungus is grown under well-defined conditions.

To study the effect of culture conditions on the immune cytokine activities, the TNF-α level was measured by treating polysaccharides obtained from the two types of bioreactors. As shown in Fig. 7, EPS obtained under the two types of bioreactors differed in their ability to induce TNF-α, whereas PPS did not seem to affect significantly TNF-α production. Therefore, culture conditions seemed to affect the properties of EPS, thereby resulting in different immune cytokine activities. This result suggests that EPS production from *L. lepideus* should be carefully controlled in fermentation to maximize their activities.

The present work showed the optimal submerged culture conditions required for the production of EPS and PPS from *L. lepideus* and compared biological activities of both types of polysaccharides obtained from various culture conditions. The culture temperature, culture period, and carbon sources in the media were the major factors that influenced the productivity of EPS and PPS. The biological activity of EPS was also influenced by culture conditions. In particular, both carbon sources and culture period affected biological activities of the EPS. The molecular mass of EPS appeared to be affected by growth kinetics. The longer it was cultured, the higher the molecular mass of EPS became. The property of PPS seemed to be stable under various culture conditions except for culture period, since the biological activity of PPS was hardly affected by culture conditions. In order to control the qualities of the products, the factors described above should be considered. The results obtained from this study could be used to identify optimal culture conditions for higher productivity and higher biological activities of polysaccharides from *L. lepideus*.

Acknowledgments

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