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Exo-Polysaccharide Production in Liquid Culture of *Pleurotus ferulae*

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Abstract Batch cultures were carried out to optimize the exo-polysaccharide production by liquid cultures of *Pleurotus ferulae*. Among the various carbon sources, when 5% of glucose was used, the maximum mycelial growth and exo-polysaccharide concentration reached were 8.78 g/l and 3.59 g/l, respectively. Yeast extract and polypeptone were identified as the most suitable nitrogen sources. In particular, when a mixture of 1% of polypeptone and 0.8% of yeast extract was used, 9.52 g/l of mycelial growth and 4.09 g/l of exo-polysaccharide were obtained. In the case of mineral sources, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ were found to be the best mineral sources for mycelial growth and exo-polysaccharide production. Under the optimized culture conditions, the agitation speed and aeration were investigated for mycelial growth and exo-polysaccharide production in a jar fermentor. The maximum mycelial growth and exo-polysaccharide concentration at 1.5 vvm and 200 rpm obtained were 13.2 g/l and 4.95 g/l, respectively, after 10 days of culture, which were 76% and 79% higher than those of the basal medium. The specific growth rate was decreased with the increase of mycelial growth. However, the specific production rate of the exo-polysaccharide was proportionally increased with the specific growth rate. The proposed model profiles showed good agreement with the experimental results for the mycelial growth and exo-polysaccharide production. The specific production rate using the optimized medium was higher than that of basal medium.

Key words: Exo-polysaccharide, *pleurotus ferulae*, liquid culture

Mushroom production has dramatically increased during recent years. The current annual world production of cultivated mushrooms is about 6.34 million metric tons, compared with only 4.92 million metric tons in 1994 [6]. From the available data, mushrooms are known to be nutritious foods, compared with vegetables, are high in protein, and have a good balance of vitamins and minerals. Mushrooms also contain little fat and digestible carbohydrates, making them suitable for low-calorie diets [11, 16]. Edible mushrooms have long been consumed by humankind since they are a high quality protein source and can be produced with higher biological efficiency than animal proteins. Besides being a non-animal protein source, several types of mushrooms are also known for their tonic and medicinal qualities. In regard to the medicinal qualities, they have been assigned significant pharmacological attributes, mainly antitumor effects [13, 14, 18, 23, 32, 34]. The term exo-polysaccharides is used to describe polysaccharides found either outside cells or free within the surrounding medium. Maziero [20] previously discussed the possibility of producing exopolysaccharides by natural Basidiomycetes from Brazil, in a submerged culture, for possible pharmacological applications, such as antitumor, antiviral, antifungal, antiparasitic, and antihistaminic activities. A number of reports on microbial exo-polysaccharide production from *Ganoderma lucidum*, *Paecilomyces japonica*, *Cordyceps militaris*, *Phellinus linteus*, and *Acremonium persicinum* are available [2, 8, 17, 26–28]. More recently, *Pleurotus ferulae*, which is a cosmopolitan group of mushrooms with a high nutritional value and therapeutic properties, has been used for various environmental and biotechnological applications. It contains about 15% of protein and the content of vitamins C, D, and E is several times higher than in other types of mushroom. [5]. Many researchers have attempted to cultivate these organisms on a solid artificial media rather than in a liquid culture [9, 19, 25, 29, 31], even though a liquid culture has potential advantages for higher mycelial production in a compact space and a shorter incubation time with a lesser chance of contamination. Furthermore, *Pleurotus ferulae*, which also

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has the ability to strengthen the immune system, can be concurrently produced.

In the present study, for effective exo-polysaccharide production from liquid cultures of *Pleurotus ferulae*, the optimum temperature, initial pH, various carbon sources, nitrogen sources, and mineral sources were first investigated in a flask. Second, under the optimized culture conditions, batch cultures were carried out in a jar fermentor. Additionally, the logistic model to describe the mycelial growth and Leudecking-Piret model for exo-polysaccharide production in a jar fermenter were proposed.

MATERIALS AND METHODS

Strain and Cultures

Pleurotus ferulae was obtained from the culture ground of Kaya-Backsong (Chungnam, Korea). Cultures were maintained on Potato dextrose agar (PDA) plates. Plates were inoculated and incubated at 25°C for 7 days, and then stored at 4°C. *Pleurotus ferulae* was initially grown on a PDA medium in a petri-dish, and then transferred into the seed medium containing malt extract 10 g/l, yeast extract 4 g/l, and glucose 4 g/l by punching out from the agar plate culture with a sterilized cork borer. The seed was grown in a 300-ml flask containing 100 ml of the seed medium at 25°C on a rotary shaker at 100 rpm for 7-8 days, and then homogenized at 10,000 rpm for 30 sec. Flask cultures for exo-polysaccharide production were carried out in 300-ml flasks containing 50 ml of the basal medium including glucose 20 g/l, yeast extract 3 g/l, malt extract 3 g/l, KH_2PO_4 1 g/l, and $MgSO_4$ 0.5 g/l, on a rotary shaker (Model HB-201SL) under specific conditions for 8°C or 12 days. All the media were sterilized at 121°C for 15 min. The pH was adjusted to the desired value by the addition of either 1 N HCl or NaOH. The culture medium was inoculated with 5% (v/v) of the mycelial homogenate and then cultivated at 25°C in a 5-l jar fermentor (Korea Fermentor Co., Korea) containing 2 l of working volume under specific conditions for 8 or 12 days. All the experiments were carried out in triplicate to ensure replication.

Analysis

The mycelial growth was obtained by centrifuging samples at 3,000 rpm for 15 min, washing the sediment three times with distilled water, and drying to a constant weight [15, 33]. All supernatants were collected, and then the crude exo-polysaccharide was precipitated with the addition of 4 volumes of 95% ethanol. The precipitated exo-polysaccharide was collected by centrifugation at 3,000 rpm for 10 min and then dried to remove the residual ethanol at 60°C. The residual glucose concentration was determined by the dinitrosalicylic acid method.

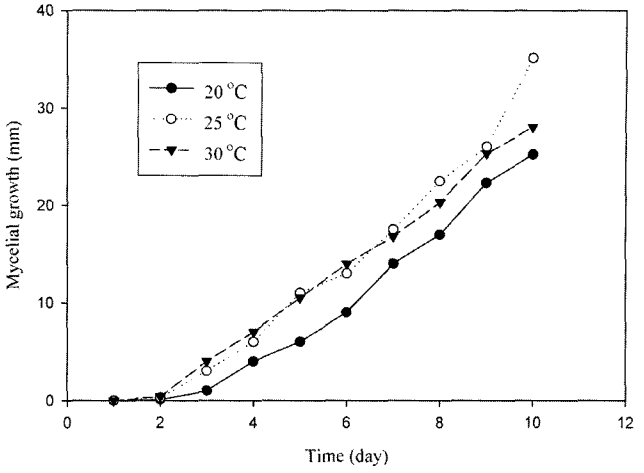

Fig. 1. Effect of temperature on *Pleurotus ferulae* growth in a flask culture.

RESULTS AND DISCUSSION

Effect of Temperature on Mycelial Growth

The effect of temperature on mycelial growth of *P. ferulae* was investigated on a petri-dish containing solid medium (YMGA) for 10 days. The culture temperature was controlled at 20, 25, and 30°C. The results are shown in Fig. 1. The mycelial growth was highest at 25°C. A similar phenomenon was also observed in *Paecilomyces japonica* [1] and *Cordyceps militaris* [20]. But in the case of *Phellinus* sp [12], the optimal temperature was 30°C.

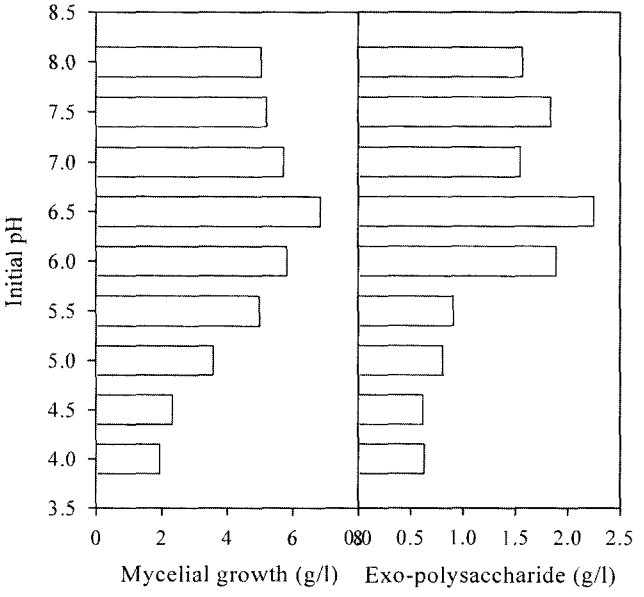

Fig. 2. Effect of initial pH on *Pleurotus ferulae* growth and exo-polysaccharide production in a flask culture.

Effect of Initial pH on Mycelial Growth and Exo-Polysaccharide Production

To investigate the effect of the initial pH on mycelial growth and exo-polysaccharide production, *P. ferulae* was cultivated in flasks in a basal medium with different initial pHs (4.0- 8.0). The mycelial and exo-polysaccharide concentrations are shown in Fig. 2. Mycelial growth and exo-polysaccharide production were highest when the initial pH ranged from 6.0 to 7.5. However, the optimal initial pH for mycelial growth was 6.5. At the initial pH 6.5, the mycelial growth and exo-polysaccharide concentrations were 6.85 g/l and 2.25 g/l, respectively. This result was similar to the result obtained by *Ganoderma lucidum* when fatty acids were used as the carbon source [30]. On the other hand, the optimum pH for mycelial growth and thioproline production in sawdust-based cultures of *Lentinus edodes* and for polysaccharide production by *Ganoderma lucidum* in glucose and ammonium chloride medium was 4.0 [22, 28] and for exo-biopolymer production by *Paecilomyces japonica* it was 5.0 [2].

Effect of Various Carbon Sources on Mycelial Growth and Exo-Polysaccharide Production

In order to investigate the effects of carbon sources on mycelial growth and exo-polysaccharide production, glucose, maltose, fructose, mannose, galactose, arabinose, xylose, lactose, sucrose, and mannitol were used. Each carbon source (2%) was added to the basal medium and tested in flasks. The results are shown in Fig. 3. When glucose, maltose, fructose, and mannose were used, the mycelial growth and exo-polysaccharide production were favorable. In particular, when glucose was used, the mycelial growth and exo-polysaccharide concentrations were 6.83 g/l and 2.75 g/l, respectively. Although the mycelial growths using maltose or fructose were high, the exo-polysaccharide production was low. Mycelial growth and exo-polysaccharide production on galactose, xylose, lactose, sucrose, and mannitol were poor. Kim *et al.* [12] reported that glucose was the best carbon source for mycelial growth and the production of the angiotensin converting the enzyme inhibitor of *Flammulina velutipes*. The pattern of exo-polysaccharide production was consistent with the mycelial growth of *P. ferulae*. These results indicated that a carbon source could be used to improve the exo-polysaccharide production in *P. ferulae*. The results showed that the good mycelial growth was closely related to the polysaccharide production. In determining the optimal concentration of glucose for the mycelial growth and exo-polysaccharide production, a range of 1 to 10% were investigated. When 5% of glucose was used, the maximum mycelial growth and exo-polysaccharide concentration reached 8.78 g/l and 3.59 g/l, respectively. When the glucose concentration was lower or higher than 5%, mycelial growth and exo-polysaccharide production were decreased (data not shown). Mikio *et al.* [21] also reported that the initial glucose concentration lower or higher than 50 g/l inhibited the mycelial growth and L-malic acid production by *Schizophyllum commune*. Therefore, it was concluded that the optimum glucose concentration for both mycelial growth and exo-polysaccharide production was 5%.

Effect of Various Nitrogen Sources on Mycelial Growth and Exo-Polysaccharide Production

To investigate the effects of nitrogen sources on the mycelial growth and exo-polysaccharide production, batch cultures were carried out in 300-ml flasks containing 50 ml of the basal medium with 0.6% of various nitrogen sources and 5% of glucose. The results are shown in Fig. 4. Among 12 different nitrogen sources, the mycelial growth and exo-polysaccharide production were very good when polypeptone and yeast extract were used. However, peptone, malt extract, and tryptone as organic nitrogen sources were

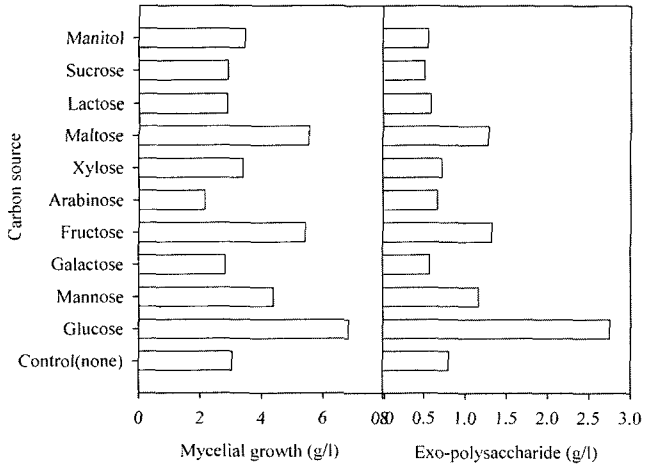

Fig. 3. Effect of carbon sources on *Pleurotus ferulae* growth and exo-polysaccharide production in a flask culture.

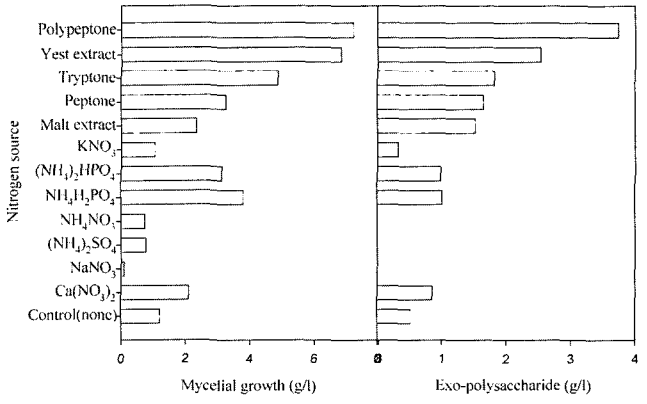

Fig. 4. Effect of nitrogen sources on *Pleurotus ferulae* growth and exo-polysaccharide production in a flask culture.

not found to be suitable for mycelial growth and exo-polysaccharide production.

Nonetheless, with inorganic nitrogen sources, the mycelial growth and exo-polysaccharide production were relatively lower than those with organic ones. The lower mycelial growth in the medium containing nitrate was similar to a common situation in fungi [10]. These results suggested that polypeptone and yeast extract seemed to contain the necessary components for the mycelial growth and exo-polysaccharide production. In order to determine the optimal concentration of polypeptone and yeast extract for mycelial growth and exo-polysaccharide production, the ranges of 0.2 to 2.0% of polypeptone and 0 to 1.0% of yeast extract were investigated. The mycelial growth and exo-polysaccharide production were increased with the increase in the polypeptone concentration up to 1.0% and then slightly decreased with a high polypeptone concentration. However, in the case of yeast extract concentration, the mycelial growth and exo-polysaccharide production was further increased with the addition of a 0.8% of yeast extract (data not shown). Therefore, the optimum concentrations of polypeptone and yeast extract were 1.0 and 0.8%, respectively.

Effect of Mineral Sources on Mycelial Growth and Exo-Polysaccharide Production

The influences of mineral sources on mycelial growth and exo-polysaccharide production in a medium containing 5% of glucose, 1.0% of polypeptone, and 0.8% of yeast extract were investigated in flasks. The mineral source was added to the medium at a concentration of 0.15%. As shown in Fig. 5, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ were found to be the best mineral sources for mycelial growth. These mineral ions are also recognized as favorable bioelements for mycelial growth and exo-polysaccharide production. The best result for the mycelial growth and exo-polysaccharide production in the medium containing K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ was also similar to the effects of various inorganic salts on enzyme production by *Aspergillus japonicus* and *Aspergillus awamoria*.

Phosphate has been used as a buffering reagent and potassium is an important mineral involving the cell structure. However, the magnesium cation can stimulate biosynthesis of the fungal cell wall and affect its permeability. When $MgCl_2$, $CaCl_2 \cdot 2H_2O$, KH_2PO_4, $MgSO_4$, Na_2HPO_4, and $FeSO_4 \cdot 7H_2O$ were used, the mycelial growth increased but the exo-polysaccharide production was significantly decreased. Also, the effect of $ZnSO_4 \cdot 7H_2O$ on the mycelial growth and exo-polysaccharide production was very poor.

Effect of Agitation on Mycelial Growth and Exo-Polysaccharide Production in a Jar Fermentor

In order to investigate the effect of the agitation speed on mycelial growth and exo-polysaccharide production, various agitation speeds were tested in a 5-l jar fermentor for 8 days. Aeration and pH were controlled at 0.5 vvm and 6.5, respectively. Figure 6 shows the time profile of the mycelial growth and exo-polysaccharide production at different agitation speeds. The agitation speed was controlled from 100 to 300 rpm and significantly affected the mycelial growth and exo-polysaccharide production. When the agitation speed was increased above 200 rpm, the mycelial growth and exo-polysaccharide production decreased. It was due to the high shear stress induced by the impeller agitation, which had a negative effect on the growth of the mycelia. The maximum mycelial growth and exo-polysaccharide production were achieved at 200 rpm of the

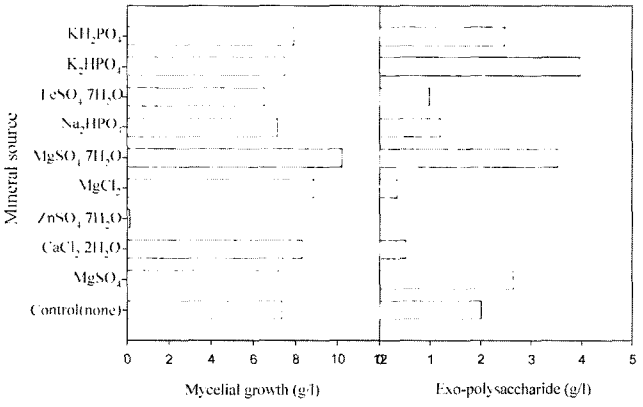

Fig. 5. Effect of mineral sources on *Pleurotus ferulae* growth and exo-polysaccharide production in a flask culture.

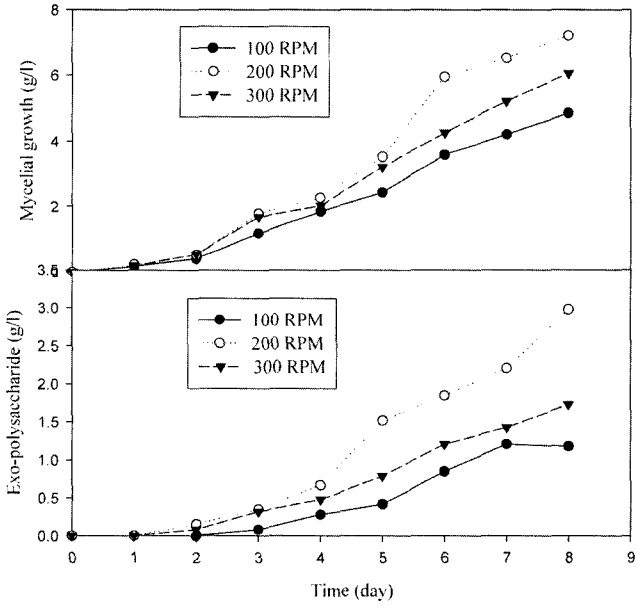

Fig. 6. Effect of agitation on *Pleurotus ferulae* growth and exo-polysaccharide production in a jar fermenter.

agitation speed, and were 7.21 g/l and 2.98 g/l, respectively. In the case of the aeration rate, the maximum mycelial growth and exo-polysaccharide production were 7.35 g/l and 3.54 g/l, respectively, when the aeration rate was 1.5 vvm (data not shown). The increase in the aeration rate resulted in the increase in the oxygen transfer rate, which led to an increase in the mycelial growth and exo-polysaccharide production.

Fermentation Under Optimal Culture Conditions

The optimal culture conditions in a jar fermentor were as follows; temperature 25°C, agitation speed 200 rpm, and aeration rate 1.5 vvm. The pH of the fermentor was controlled at 6.5. Based on the results of the flask cultures, a batch culture using the optimized medium in a jar fermentor was carried out for 12 days. Figure 7 shows the change of *P. ferulae* mycelial growth, exo-polysaccharide production, and residual glucose concentrations. The mycelial growth and exo-polysaccharide production were the highest after 10 days of cultivation and were 13.2 g/l and 4.95 g/l, respectively. The mycelial growth rate of *P. ferulae* was rapidly increased for the first 4 days and the sugar consumption decreased rapidly. The residual glucose concentration decreased from 50 to 23.8 g/l during the cultivation. These results indicated that exo-polysaccharide production increased in parallel with the mycelial growth and that the product formation was associated with mycelial growth.

Development of Mycelial Growth and Exo-Polysaccharide Production Model

A logistic model to describe the mycelial growth and a Leudecking-Piret model for the exo-polysaccharide production in a 5-l jar fermentor.

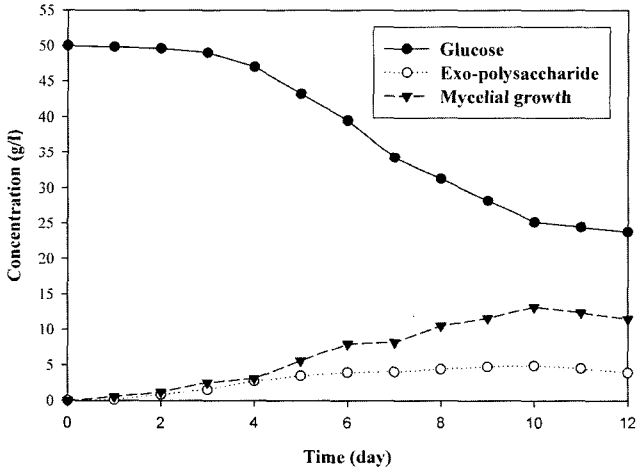

Fig. 7. Changes of *Pleurotus ferulae* growth, and exo-polysaccharide and glucose concentrations in a jar fermenter under optimal medium composition.

$$dX/dt=\mu X \quad (1)$$

$$\mu=\mu_{max}(1-X/X_m) \quad (2)$$

$$dP/dt=\alpha dX/dt+\beta X \quad (3)$$

where X is the mycelial growth, P is the exo-polysaccharide concentration, μ is the specific mycelial growth rate, α is the growth-associated product formation coefficient, and β is the nongrowth-associated product formation coefficient. The data in Fig. 7 were used to estimate model parameters, μ_{max}, X_m, α, and β. The specific mycelial growth rate decreased with the increase of mycelial growth and their

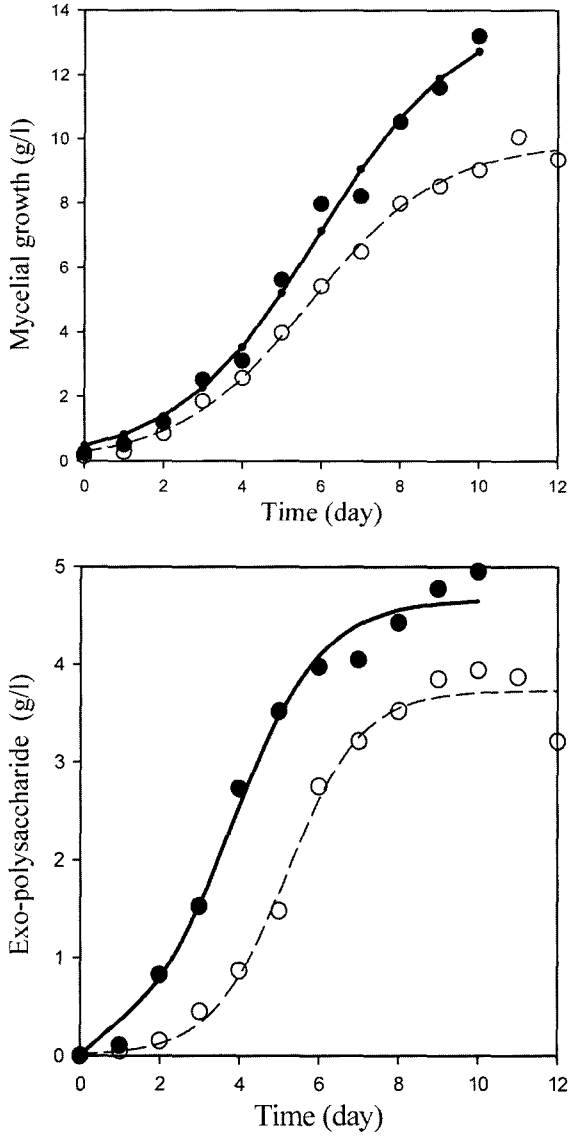

Fig. 8. Comparison between basal media and developed media composition on mycelial growth and exo-polysaccharide concentration.

Symbols: ●, experimental data with developed media; ○, experimental data with basal media; —: simulation data with developed medium; ---: simulation data with basal medium.

Table 1. List of estimated parameters.

Parameter	Basal media	Developed media
μ_{max}	0.57	0.54
X_m	9.5	13.2
α	0.87	1.59
β	0	0

correlation can be expressed as Eq. (2). The μ_{max} and X_m determined with regression analysis are 0.57/day and 9.5 g/l, respectively. α and β are estimated from the correlation between specific growth rate and specific production rate. The specific production rate proportionally increased with specific growth rate. The slope, α, was 0.87, and the exo-polysaccharide was not produced when the cells were in a stationary phase. Therefore, we concluded that the exo-polysaccharide production depended on only the mycelial growth and β is zero. Figure 8 shows the experimental data and simulated results with the developed model using the optimum medium and basal medium. The developed model showed good agreement with the mycelial growth and exo-polysaccharide production. The mycelial growth and exo-polysaccharide production model under the optimal medium composition were developed. The estimated parameters using the developed medium were 0.54/day, 13.2 g/l, 1.59, and 0 for μ_{max}, X_m, α, and β, respectively. Table 1 shows the comparison of model parameters using a jar fermentation with optimal medium and basal medium. With optimal medium, the specific mycelial growth rate was higher than that with basal medium. Since the exo-polysaccharide was only produced with the mycelial growth, it followed a growth-associated production. Exo-polysaccharide was not produced when the cells were in the stationary phase. The production of the growth-associated exo-polysaccharide increased by 79% with the optimal medium.

The production of polysaccharides by liquid culture reduced the costs and time, while production was increased and controlled. Furthermore, the biomass produced in a submerged culture can be directly used as inoculum in the mushroom production process, as a source for metabolic intermediates production, or as food complements [7]. The optimizations of a liquid culture for effective mycelia growth and exo-polysaccharide production using *Pleurotus ferulae* were carried out. The optimal temperature for *Pleurotus ferulae* growth was 25°C and the optimal initial pH for mycelial growth and exo-polysaccharide production was 6.5. Among the 10 carbon sources examined, glucose, maltose, fructose, and mannose were all favorable for mycelial growth and exo-polysaccharide production. Specifically, the maximum mycelial growth and exo-polysaccharide concentration were achieved in the medium containing 5% of glucose. In the case of a nitrogen source, polypeptone and yeast extract were both very good for the mycelial growth and exo-polysaccharide production. In the case of mineral sources, K_2HPO_4 (0.12%) and $MgSO_4 \cdot 7H_2O$ (0.12%) were the most effective inorganic salts for mycelial growth and exo-polysaccharide production. Using the optimized medium, the agitation speed and aeration were investigated for effective mycelial growth and exo-polysaccharide production in a jar fermentor. When agitation speed was increased above 200 rpm, mycelial growth and exo-polysaccharide production decreased due to higher shear stress induced by the impeller agitation. The maximum mycelial and exo-polysaccharide concentrations after 10 days of culture were 13.2 g/l and 4.95 g/l, respectively, when the aeration rate was 1.5 vvm at 200 rpm of the agitation speed. The mycelial growth and exo-polysaccharide production using the optimized medium were 76% and 79%, respectively higher than those of the basal medium. We proposed the logistic model to describe the mycelial growth and Leudecking-Piret model for the exo-polysaccharide formation in *P. ferulae*. The specific growth rate was decreased with the increase of the mycelial growth. However, in the case of the specific production rate of the exo-polysaccharide, this was proportionally increased with the specific growth rate. The model profile showed good agreement with the experimental results for mycelial growth and exo-polysaccharide production. The specific production rate using the modified medium is higher than that of basal medium. To achieve and control the optimal morphology, the relation between the operational conditions and fungi must be known [7, 24]. Thus, more work on the possible relations among the morphology, growth, and exo-polysaccharide production are needed. To meet the requirements of large-scale exo-polysaccharide production, further studies are also needed, including the configuration of a suitable reactor, optimization of the culture conditions, and characterization of the exo-polysaccharide produced by *P. ferulae*.

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