Korean J. Chem. Eng., **28**(6), 1427-1432 (2011)
DOI: 10.1007/s11814-011-0109-2

A study of mycelial growth and exopolysaccharide production from a submerged culture of *Mycoleptodonoides aitchisonii* in an air-lift bioreactor

DuBok Choi*, Jun Han Lee, Yun-Soo Kim**, Myung-Sun Na***, On-You Choi****, Hee-Duck Lee*****, Myung Koo Lee*, and Wol-Suk Cha**,†**

*Department of Pharmacy, College of Pharmacy, Chungbuk National University, Cheongju 361-763, Korea
**Department of Biochemical and Chemical Engineering, Chosun University, Gwangju 501-759, Korea
***Department of Beauty and Cosmetic, Chosun University, Gwangju 501-759, Korea
****Department of Environmental Health, Cho-dang University, Jeonnam 534-800, Korea
*****Korea Advanced Food Research Institute, Seoul 137-850, Korea
(*Received 19 December 2010 • accepted 24 April 2011*)

Abstract−We determined the optimal culture and medium conditions for effective production of mycelial mass and exopolysaccharide from a liquid culture of *Mycoleptodonoides aitchisonii* in an air-lift bioreactor. The mycelial growth and exopolysaccharide production were found to be optimal at a temperature of 25 °C and pH of 6.5. When 60 g/L of lactose was used as a carbon source, the maximum mycelial growth and exopolysaccharide production were obtained. The polypeptone and yeast extract were the most appropriate nitrogen sources for mycelial growth and exopolysaccharide production. In addition, when a mixture of 20 g/L of polypeptone and 5 g/L of yeast extract was used, the exopolysaccharide production increased 50% compared to that of the sole nitrogen source. $CaCl_2 \cdot 2H_2O$ (1.0 g/L) was the most effective mineral source. Using the optimal culture and medium conditions, batch cultures with basal and designed medium on mycelial growth and exopolysaccharide production in a 5 L air-lift bioreactor were carried out for 16 days. The mycelial growth and exopolysaccharide production increased with an increase of culture time at 14 days, and the maximum mycelial growth and exopolysaccharide production were 20.3 and 6.2 g/L, respectively, after 14 days of culture. The developed model in an air-lift bioreactor showed good agreement with experimental data. These results indicate that exopolysaccharide production is associated with the mycelial growth of *M. aitchisonii* in an air-lift bioreactor.

Key words: Exopolysaccharide, Liquid Culture, *Mycoleptodonoides aitchisonii*, Air-lift Bioreactor

INTRODUCTION

Mushrooms are abundant sources of a wide range of useful natural products and serve as delicacies for human consumption and as nutriceuticals, or "foods that also cure." They contain substances of various kinds that are highly valued as medicines, flavorings, and perfumes. Mushroom growing has a long tradition in Eastern Asian countries, especially in China where it started circa 600 A. D. with *Auricularia auricular*, the Wood Ear. In Europe, cultivation of *Agaricus bisporus*, the Button Mushroom was first achieved in France during the seventeenth century [1].

Mycoleptodonoides aitchisoni belongs to the Climacodontaceae family and has been widely cultivated on dead broad-leaf trees from summer to fall in Asia. There is no pileus on the fruiting body. Its cap is fan- or spatula-shaped. The size is approximately 3×3 to 8×10 cm. The surface is smooth, the edge is tooth-shaped, and the mushroom is a white or straw color. The body is white, 2-5 mm thick, and smells like butylacetate ester. The mycelium consists of two needle-shaped mycelia approximately 3-10 mm. The spore size is approximately 2-2.5×5-6.5 μm [2]. Tsuchida et al. reported that an aqueous extract from *M. aitchisonii* improved hypertension, without showing subjective symptoms or affecting haematological or urinalytical parameters [3]. Okuyama et al. examined the effects of *M. aitchisonii* extract on the synthesis of nerve growth factor and neurotransmitter metabolism in the brain of Wistar rats fed a controlled diet, and they suggested that *M. aitchisonii* has an enhancing effect on the synthesis of nerve growth factor and catecholamine metabolites in the rat brain [2]. Okuyama et al. isolated derivatives of linalool or a fragrant conductor compound, phenylpentane, which contains 1-phenyl-3-pentanol and 1-phenyl-3-pentanone, from *M. aitchisonii* and investigated dopamine release using rat striatal slices [4]. Also, endoplasmic reticulum stress-protecting compounds were isolated from *M. aitchisonii* and showed protective activity against endoplasmic reticulum stress-dependent cell death [5].

Many researchers have tried to culture mushrooms on solid artificial media in order to obtain polysaccharide and mycelial biomass. However, this method does not guarantee identical substituents from batch to batch. As a result, attention has been paid to the use of liquid culture for the large-scale production of mycelial biomass and polysaccharide. A liquid culture using mushrooms has potential advantages for higher mycelial production in a compact space and for a shorter incubation time with a smaller chance of contamination. We previously investigated the liquid cultures for effective mycelia growth and polysaccharide production from *Pleurotus ferulae*, *Lentinus* sp., *Pleurotus nebrodensis*, and *Agrocybe aegerita* in a jar fermentor [6-9]. We also are attempting to confirm the possibility of enhancing the production of mycelial biomass and polysaccharide production using an air-lift bioreactor, because air-lift bioreactors have many economic implications for reactor construction,

†To whom correspondence should be addressed.
E-mail: wscha@mail.chosun.ac.kr

maintenance, and scale up [10].

In this study, to achieve the high mycelial growth and exopolysaccharide production in the liquid culture of *M. aitchisonii* in the air-lift bioreactor, various environmental factors affecting mycelial growth and polysaccharide production were investigated in flask cultures. Using the optimal culture and medium conditions, batch cultures using basal and designed medium on mycelial growth and exopolysaccharide production were carried out in a 5 L air-lift bioreactor. In addition, kinetic study of mycelial growth and exopolysaccharide production in an air-lift bioreactor was carried out.

MATERIALS AND METHODS

1. Strain and Cultures

Mycoleptodonoides aitchisonii was obtained from the culture ground of Kaya-Backsong (Chungnam, Korea). Plates were incubated at 25 ºC for 10 days and stored at 4 ºC. *M. aitchisonii* was initially grown on a potato dextrose agar medium in a petri-dish and transferred into the seed medium containing potato dextrose broth by punching out from the agar plate with a sterilized cork borer. The seed was grown in a 300 mL flask containing 50 mL of the seed medium at 25 ºC on a rotary shaker at 100 rpm for 2 days, and the culture broth with mycelia and spores was homogenized aseptically in an omni-mixer for 3 min in an ice bath. About 5% of the mycelial homogenate was inoculated in 5 L of an air lift bioreactor containing culture media and cultivated at 25 ºC. All experiments were conducted in triplicate to ensure reproducibility.

2. Analysis Method

Mycelial growth was obtained by centrifuging sample at 3,000 rpm for 10 min and filtering through a pre-weighed filter (Whatman GF/C Cat No. 1822047). The obtained filter was washed twice with distilled water and dried at 90 ºC before measuring the weight of mycelia. All supernatants were collected, and the crude exopolysaccharide was precipitated with addition of 95% ethanol. The precipitated exopolysaccharide was collected by centrifugation at 3,000 rpm for 10 min and dried to remove residual ethanol at 60 ºC. Residual glucose or lactose concentration was determined by the dinitrosalicylic acid method.

3. Air-lift Bioreactor

The air-lift bioreactor is composed of three parts: a conical bottom holding the sparger, a cylindrical middle section, and a top portion with a degassing zone. To increase the mixing characteristics, the draft tube was removed and replaced with four ring spargers and wire nets. An air-lift bioreactor has several ports for measuring the dissolved oxygen concentration and foam inside the bioreactor, for removing exhaust gas, and for adding antifoam agents; it also has a sampling port at the bottom of the cylindrical section. The temperature of the contents of an air-lift bioreactor can be controlled by circulating water through a jacket.

RESULTS AND DISCUSSION

1. Effect of Culture Temperature on Mycelial Growth and Exopolysaccharide Production

The effects of culture temperature on mycelial growth and exopolysaccharide production in the culture f *M. aitchisonii* were investigated for six days in a flask. The culture temperatures were controlled at 20, 22.5, 25, 27, and 30 ºC. The results are shown in Fig. 1. The mycelial growth was similar up to two days of culture regardless of culture temperatures. However, it increased with an increase of culture time after four days of culture (data not shown). The maximum mycelial growth and exopolysaccharide production were obtained, 3.2 and 1.0 g/L at 25 ºC after six days of culture. However, above or below 25 ºC, it was decreased. This result indicates that mycelial growth and exopolysaccharide production form *M. aitchisonii* were significantly affected by culture temperature. Similar results also were observed in the culture of *Pleurotus eryngii*, *Lentinus lepideus*, *Naematoloma Sublateritiu*, and *Phellinus linteus* [11, 12]. In the case of *Fammulina veltipes*, the optimal temperature for mycelial growth was 30 ºC, but the production of angiotensin converting enzyme inhibitor at 30 ºC was lower than that of the culture at 20 ºC [13]. The optimal temperature for mycelial growth in fed-batch culture of *Ganoderma resinaceum* DG-6556 was 28 ºC, whereas the maximum exopolysaccharide production was achieved at 31 ºC [14]. For *Cordyceps militaris*, the optimum temperature on the mycelial growth and exobiopolymer production was 20 ºC [15]. In the case of *Hericium erinaceus* for the exopolysaccharide production in liquid culture, it was 23 ºC [16]. The optimum temperature for adsorption and decolorization of dyes using solid residues from *Pleurotus ostreatus* was in the range of 26-36 ºC [17].

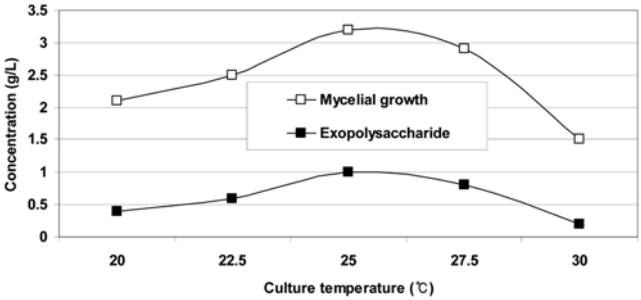

Fig. 1. Effect of temperature on mycelial growth and exopolysaccharide production.

2. Effect of pH on Mycelial Growth and Exopolysaccharide Production

Many researchers reported that cell membrane function, cell growth cell morphology and structure, solubility of salts, ionic state of substrate, uptake of various nutrients, and product biosynthesis were

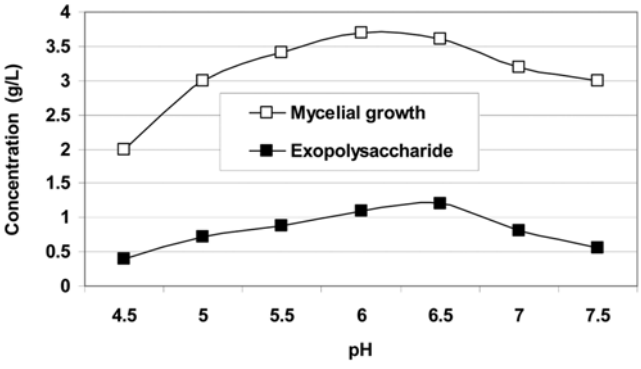

Fig. 2. Effect of pH on mycelial growth and exopolysaccharide production.

affected by the pH of the medium [18-20]. To investigate the effects of the initial pH of medium on mycelial growth and exopolysaccharide production at 25 °C, batch cultures were carried out within a pH range of 4.5 to 7.5 for 6 days. The results are shown in Fig. 2. When the cultures were maintained within a pH of 6.0 to 6.5, the optimal growth rates were obtained. However, at pH above 7.0 or below 5.0, they were decreased. Conversely, in exopolysaccharide production, optimal exopolysaccharide production was obtained in the pH range of 5.5 to 6.5. However, at pH above 7.5 or below 5.0, production was decreased. Maximum mycelial growth at pH 6.0 and exopolysaccharide production at pH 6.5 were obtained with 3.8 and 1.3 g/L, respectively. These results are similar to that of *Cordyceps jiangxiensis* JXPJ 0109 [18]. In *Sphaeropsis pyriputrecens*, it grew at pH 3.0-6.0, and optimum growth was at pH 3.2-4.2. However, no mycelial growth resumed above pH 8.0 [21]. The optimal pH for mycelial growth and exopolysaccharide production from *Phellinus linteus*, *Poria cocos*, *Coriolus versicolor*, and *Grifola frondosa* was 5.0 [1,22,23]. The optimal pH for mycelial growth of *Lentinus lepideus* was 4.2. However, no mycelial growth resumed above pH 5.0 [24]. The optimal mycelial growth for *Formitella fraxinea* was at pH 6.0 [25], *Pleurotus ostreatus* was at pH 6.2-6.5 [26], *Lentinus edoes* was at pH 4.0-4.5 [27], *Poria cocos* was at pH 4.0 [28], *Phelinus linteus* was at pH 4.2 [29], and *Pleurotus eryngii* was at pH 6.0 [30]. These results are similar to optimal pH ranges (pH 4.0-7.0) of mycelial growth of Basidiomycetes [31].

3. Effect of Carbon Source on Mycelial Growth and Exopolysaccharide Production

Nutritional requirements for mycelial growth and exopolysaccharide production in basidiomycetes and ascomycetes depend on strains and culture conditions. In addition, different carbon sources can result in different carbohydrate compositions in the polysaccharides produced [32]. To select the best carbon source for mycelial growth and exopolysaccharide production, glucose, maltose, fructose, arabinose, lactose, and manitol were investigated for eight days. Each carbon source (60 g/L) was added to the basal medium and tested in flasks. The results are shown in Fig. 3. When glucose, maltose, fructose, or lactose was used, the mycelial growth and exopolysaccharide production were favorable. In particular, when lactose was used, the maximum mycelial growth and exopolysaccharide concentrations were obtained, 4.98 and 1.5 g/L, respectively. However, the mycelial growth and exopolysaccharide production were poor when mannitol or arabinose was used. The pattern of exopolysaccharide production was consistent with mycelial growth. The results showed that strong mycelial growth was closely related to polysaccharide production. In the case of mycelial growth and exopolysaccharide production from *Ganoderma resinaceum*, *Coriolus versicolor*, *Pasthyerella atroumbonata*, *Cordyceps militaris*, *Fomitopsis pinicola*, *Lentinus edodes*, *Hericium erinaceus*, and *Tricholoma matsutake*, glucose was the most suitable carbon source [15, 23,33-36]. However, the maximum mycelial growth and exopolysaccharide production in the culture of *Paecilomyces japonica* using maltose [37], *Ganoderma lucidum* using xylose, sorbitol and manitol, *Daedalea aquercina* using dextrin [38], and *Pacilomyces japonica* using maltose and sucrose [39] were obtained. In the case of *Termitomyces clypeatus* for production of both cellulolytic and xylanolytic enzymes in a submerged culture, tamarind kernel powder was also used [40].

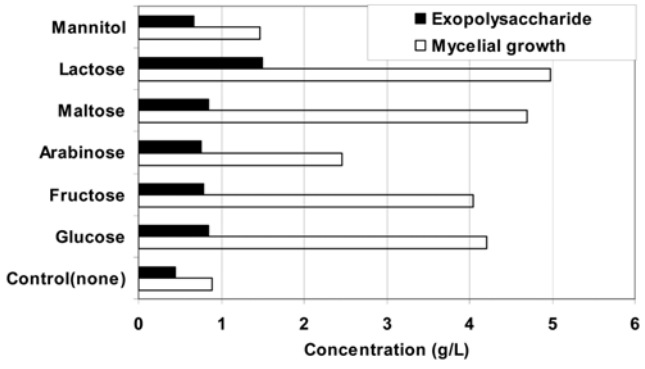

Fig. 3. Effect of carbon source on mycelial growth and exopolysaccharide production.

4. Effect of Nitrogen Source on Mycelial Growth and Exopolysaccharide Production

Nitrogen is one of the primary components of living materials, and it plays a key role in biological regulation. Nitrogen sources can govern cellular growth, formation of products, and cellular enzymes. The regulatory effects of nitrogen on the production enzymes involved in nitrogen assimilation, catabolism of nitrogenous compound, and formation of glutamate have been reviewed [41]. To investigate the effects of nitrogen sources on the mycelial growth and exopolysaccharide production, batch cultures were maintained for ten days in flasks containing 60 g/L of lactose at 25 °C with various organic and inorganic nitrogen sources (2%), such as $(NH_4)_2HPO_4$, KNO_3, $Ca(NO_3)_2 \cdot 4H_2O$, yeast extract, malt extract, beef extract, peptone, polypeptone, and tryptone. The results are shown in Table 1. When organic nitrogen sources were used, mycelial growth and exopolysaccharide production increased compared to inorganic nitrogen sources. In particular, the highest mycelial growth and exopolysaccharide production were achieved in media containing polypeptone and yeast extract. Mycelial growth was closely related to exopolysaccharide production in the culture of *Coriolus versicolor* [42]. These results suggest that polypeptone and yeast extract might contain the components necessary for mycelial growth and exopolysaccharide production. However, mycelial growth and exopolysaccharide production of *Ganoderma lucidum* using inorganic nitrogen sources were similar to those for organic nitrogen sources [43].

Table 1. Effect of nitrogen source on mycelial growth an exopolysaccharide production

Nitrogen source	Mycelial growth (g/L)	Exopolysaccharide (g/L)
Control(none)	1.5	0.5
$(NH_4)_2HPO_4$	0.72	0.53
KNO_3	0.83	0.56
$Ca(NO_3)_2 \cdot 4H_2O$	2.64	0.98
Yeast extract	4.67	1.4
Malt extract	3.39	1.01
Beef extract	1.04	0.6
Peptone	2.52	0.97
Poly-peptone	6.78	1.6
Tryptone	2.6	0.97

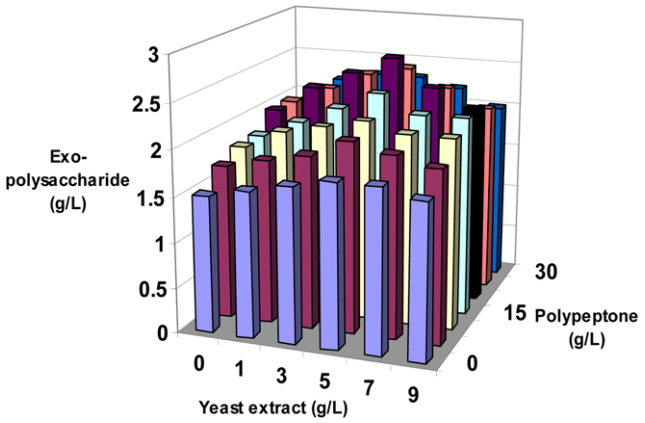

Fig. 4. Effect of the mixing ratio of yeast extract and polypeptone on exopolysaccharide production.

To determine the optimal mixing ratio of polypeptone and yeast extract for obtaining a high level of the exopolysaccharide production, batch cultures were carried out for 12 days in flasks. A total of 42 nitrogen sources were tested, and the results are shown in Fig. 4. The exopolysaccharide concentration increased with the increase of polypetone concentration. The exopolysaccharide production was strongly affected by polypetone concentration. When polypeptone concentration increased from 5.0 to 20 g/L, it was increased from 1.2 to 2.0 g/L. But at above 25 g/L, it did not increase. Conversely, when yeast extract concentration increased from 1.0 to 5.0 g/L, it was increased from 1.1 to 1.8 g/L. But it also did not improve at above 7.0 g/L. The values for a mixture of polypeptone and yeast extract increased compared to those for sole polypeptone or yeast extract. Therefore, the optimal mixing ratio was 5.0 g/L of yeast extract and 20 g/L of polypeptone, which yielded an exopolysaccharide concentration of 2.7 g/L.

5. Effect of Mineral Source on Mycelial Growth and Exopolysaccharide Production

To investigate the effect of mineral source on mycelial growth and exopolysaccharide production in a medium containing 60 g/L of lactose, polypeptone (20 g/L), and yeast extract (5 g/L) at 25 °C, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$, NaCl, and KCl were used, and each mineral source was added to the medium at a concentration of 0.15%. The results are shown in Table 2. $FeSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$ were the optimal mineral sources for mycelial growth and exopolysaccharide production. When 0.1% of $CaCl_2 \cdot 2H_2O$ was used, mycelial growth and exopolysaccharide production were 9.4 and 3.0 g/L, respectively, after 14 days of culture. However, when $MnCl_2 \cdot 4H_2O$, NaCl, or KCl was used, the mycelial growth and exopolysaccharide production ranged from 3.8 to 4.2 g/L and 2.0 to 2.2 g/L, respectively. Predominantly, these results are similar to that of control. However, for $MnCl_2 \cdot 4H_2O$, the mycelial growth and exopolysaccharide production were 3.1 and 1.9 g/L, respectively. Jonathan and Fasidi [44] reported that Mg^{2+}, K^+, and Ca^{2+} promoted mycelial growth of *L. subnudus* and *S. commune*. In *Pellinus* sp., KH_2PO_4 and $CaCl_2$ were the most effective mineral sources for mycelial growth [45]. In *P. ferulae* and *Pleurotus nebrodensis* Inzenga, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ were the best mineral sources for mycelial growth and exopolysaccharide production (data not shown).

Table 2. Effect of mineral source on mycelial growth and exopolysaccharide production

Mineral source	Mycelial growth (g/L)	Exopolysaccharide (g/L)
Control (none)	4.95	1.8
$MgSO_4 \cdot 7H_2O$	4.46	2.19
$FeSO_4 \cdot 7H_2O$	7.44	2.32
$CaCl_2 \cdot 2H_2O$	9.3	3
$MnCl_2 \cdot 4H_2O$	3.14	1.94
NaCl	4.19	2.11
KCl	3.76	2.03

6. Batch Culture for Mycelial Growth and Exopolysaccharide Production in an Air-lift Bioreactor

Batch cultures using basal medium containing glucose (60 g/L), yeast extract (5 g/L), and $MgS0_4$ (0.15 g/L) and designed medium containing lactose (60 g/L), polypeptone (20 g/L), yeast extract (5 g/L), and $CaCl_2 \cdot 2H_2O$ (1.0 g/L) in a 5 L air-lift bioreactor containing 2.5 L of working volume at 25 °C and 1.0 vvm for 16 days were compared. The changes of mycelial growth, exopolysaccharide production, and residual glucose or the lactose concentrations are shown

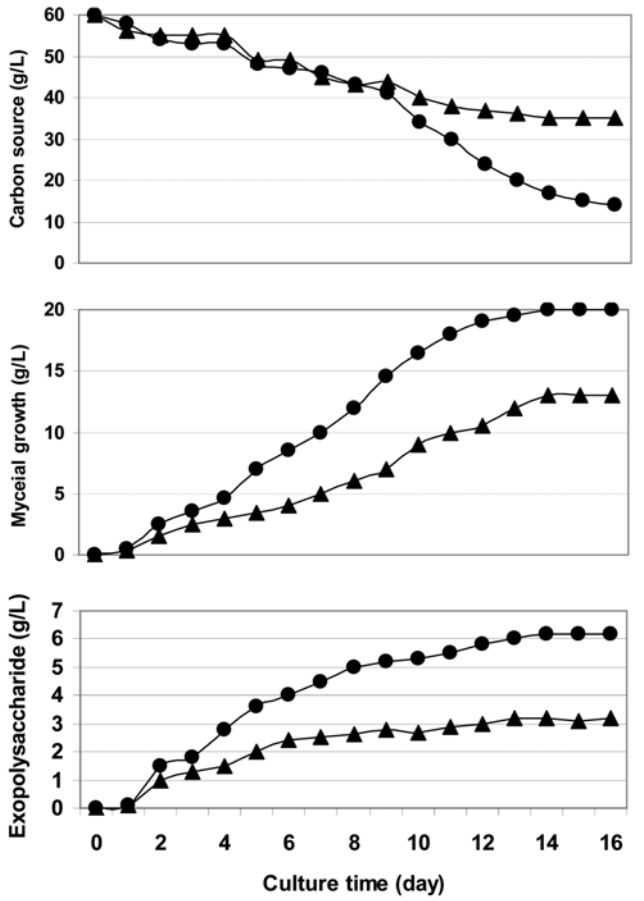

Fig. 5. Comparison of basal medium and designed medium on carbon consumption, mycelial growth, and exopolysaccharide production in an air-lift bioreactor. The (▲) represents basal medium, and the (●) represents designed medium.

in Fig. 5. When the basal medium was used, the glucose was consumed with an increase of culture time up to 14 day of culture, but consumption did not increase after 15 days of culture. The glucose consumed was 25.50 g/L after 14 days of culture. Mycelial growth increased with an increase of culture time up to 13 days of culture, 11.2 g/L and did not increase after 14 days of culture. Exopolysaccharide production also increased with an increase of culture time and it was 3.4 g/L after 14 days of culture. Conversely, when the designed medium was used, lactose consumed increased with an increase of culture time for 14 days of culture but did not increase after 15 days of culture. The lactose consumed was 44.60 g/L after 14 days of culture. Mycelial growth increased with culture time for the initial 14 days of culture, and the maximum mycelial growth was 20.3 g/L after 14 days of culture, which was approximately 1.5-fold higher than that of basal medium. The maximum concentration of exopolysaccharide was 6.2 g/L, which was approximately 2.1-fold higher than that of basal medium after 14 days of culture. These results indicate that exopolysaccharide production increased in parallel with growth of mycelium, and product formation is associated with mycelial growth in an air-lift bioreactor using *M. aitchisonii.* It is difficult to culture aerobic fungi in an air-lift bioreactor because of the mycelial morphology, which results in an increase in apparent viscosity due to mycelial growth or medium and unplanted mixing. However, mycelial growth and exopolysaccharide production in the culture of *M. aitchisonii* after refinement of the medium and culture conditions were similar to that of a jar fermentor (data not shown). This suggests that an air-lift bioreactor has potential for use in exopolysaccharide production and mycelial growth using *M. aitchisonii*. Since it is a high yield exopolysaccharide producer, is simple, and uses an inexpensive production medium, the air-lift bioreactor may lead to realization of large scale production of exopolysaccharide.

7. Kinetics of Mycelial Growth and Exopolysaccharide Production in an Air-lift Bioreactor

Kinetic studies supported by mathematical models are a vital part of the overall investigation of cell growth and product formation in the culture process. The model allows easy data analysis and provides a strategy for solving problems encountered at the design stage. Knowledge and an understanding of the kinetics of exopolysaccharide production are of great economic importance because exopolysaccharide production is important in many culture processes [46]. Recently, kinetic studies for several extracellular microbial polysaccharides in liquid static cultures of *Ganoderma lucidum*, *Aureobasidium pullulans*, and *Pleurotus ferulae* have been reported [47-49]. Despite a great need for the development of mushroom cultivation processes for efficient production of useful metabolites, until now there has been no report of their use in bioprocess modelling for large-scale cell growth and exopolysaccharide production with liquid culture of *M. aitchisonii* in an air-lift bioreactor. The logistic equation was used to fit the mycelial growth curves. A common autonomous rate equation is the differential form of the logistic equation:

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

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

Where X, X_m, μ, and μ_{max} are the mycelial growth, maximum mycelial growth, specific mycelial growth rate, and maximum specific mycelial growth rate, respectively. The Leudecking-Piret equation developed for exopolysaccharide production was applied:

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

Where P, α, and β are the exopolysaccharide concentration, growth-associated product formation coefficient, and nongrowth-associated product formation coefficient, respectively.

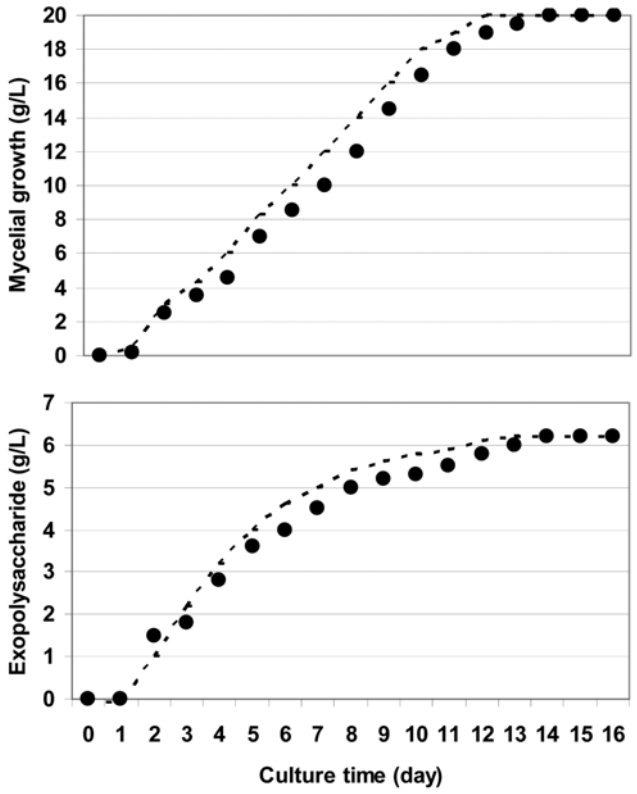

Fig. 6. Comparison of experimental and simulation data of mycelial growth and exopolysaccharide concentration. The (●) represents experimental data, and the dotted line (------) represents the simulation result.

Fig. 6 shows the experimental data and simulated results from the developed model using the optimal media. The developed model showed good agreement with mycelial growth and exopolysaccharide production. The data from the developed media culture were used to estimate model parameters μ_{max}, X_m, α, and β. μ_{max} and X_m determined by regression analysis using Eq. (2) were 0.64 g/day and 19.8 g/L, respectively. α and β were estimated from the correlation between specific growth rate and specific production rate using Eq. (3). The slope, α, was 0.90, and β was zero. This result suggests that the model can describe the liquid culture process of *M. aitchisonii* in an air-lift bioreactor.

CONCLUSIONS

For efficient mycelial growth and exopolysaccharide production from submerged culture of *M. aitchisonii* in an air-lift bioreactor, various environmental factors affecting the mycelial growth and exopolysaccharide production were investigated. The optimal tem-

perature and pH were 25 ºC, and 6.5, respectively. Lactose (60 g/L), polypeptone (20 g/L), yeast extract (5 g/L), and $CaCl_2 \cdot 2H_2O$ (1.0 g/L) were the most effective energy sources for mycelial growth and exopolysaccharide production. Using the optimal culture condition and medium composition, batch cultures were carried out in a 5 L air-lift bioreactor. The maximum concentration of exopolysaccharide was approximately 2.1-fold higher than that of basal medium after 14 days of culture. The developed model for an air-lift bioreactor showed good agreement with experimental data and simulated results for mycelial growth and exopolysaccharide production in a culture of *M. aitchisonii*. However, the fundamental information obtained from this study is insufficient for the development of an efficient process for mycelial growth and exopolysaccharide production from *M. aitchisonii* in an air-lift bioreactor. To meet the requirements of large-scale mycelial growth and exopolysaccharide production in an air-lift bioreactor, therefore, further studies are needed.

ACKNOWLEDGEMENTS

This research was financially supported by Chosun University in 2010.

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