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Effect of Ammonium Phosphate on Mycelial Growth and Exopolysaccharides Production of *Ganoderma lucidum* in an Air-Lift Fermenter

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Abstract It was discovered that ammonium phosphate in the medium played an important role in both growing mycelium and producing exopolysaccharides (EPS) from *G. lucidum*. In lower concentration levels of ammonium phosphate (0–3 g/l), an improved mycelial growth was observed by maintaining more filamentous morphology than in high concentrations (5–11 g/l). In addition, it was confirmed by comparing the factual dimension and frequency of the area regarding the mycelial pellets. This must be attributed to limitations of nutrient transfer by maintaining filamentous mycelium during the cultivation in a low ammonium phosphate containing medium. On the other hand, the best EPS production was observed in medium with the absence or low concentration of ammonium phosphate. The shear stress of the culture broth was greatly affected by the shear rate, as compared with that of the culture broth with high ammonium phosphate concentration. The rheological characteristics of the fermentation broth and filtrate worked well according to the Herschel-Bulkley model. It was also found that the morphological changes of the mycelium resulting from the ammonium phosphate concentration directly affected the rheological characteristics of the system and resulted in reversely affecting the EPS production levels. Based on these results, it can be concluded that delicate regulation of the ammonium phosphate concentration in the culture media should be provided in order to obtain optimal mycelial growth and/or EPS production.

Key words: Ammonium phosphate, morphology and viscosity, mycelial cultivation, *Ganoderma lucidum*

Mushrooms have attract a great deal of interest in many areas of foods and biopharmaceuticals, etc. [7, 14]. The main components used for biotechnological applications are polysaccharides, which exist within the mushrooms or in its secretion [12, 18]. Recently, exopolysaccharides (EPS) have been investigated extensively, because their production processes from culture broth do not require extra steps and they require relatively simple purification processes [5, 7, 20]. However, it has been very difficult to culture large amounts of mycelium in a large scale fermenter in order to produce reasonable amounts of EPS. There have been several limitations in scaling-up the mycelial culture system due to its low secretion level and the change in the characteristics of the culture broth such as viscosity, morphology, etc. [3, 9]. A major problem in cultivating the mycelium is the increase of viscosity of the culture broth as the mycelium grow, which results not only in limiting oxygen transfer, but also in building up shear stress. High shear stress has a negative effect on the morphology of the mycelium which can directly reduce the cell growth along with the polysaccharide production [15]. No attempts have been made to show the effect of adding ammonium phosphate in previous works even though while growing the cells in ammonium phosphate containing medium [10, 11]. For filamentous fungi, it is a well documented phenomenon that cell growth was increased by adding ammonium ions, possibly due to carbon accumulations by the cell [4]. However, a repressive effect of excess ammonium ions on antibiotic production has also been reported [5]. Therefore, in this study, we investigated the effect of ammonium phosphate on both mycelial growth and EPS production from *Ganoderma lucidum* for enhancing pharmaceutical efficacy [13]. The role of ammonium phosphate for rheology and morphology during cultivation of *G. lucidum* was also examined.

Materials and Methods

Organisms and Culture Conditions

The stock culture of *Ganoderma lucidium* ASI 7004 was maintained on potato dextrose agar (PDA) plate at 24°C. The seed culture was cultivated in an optimized medium

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containing glucose 60 g/l, yeast extract 6 g/l, $(NH_4)_2HPO_4$ 5 g/l, and KH_2PO_4 0.5 g/l [10]. An initial pH was adjusted to 3.0 by 1 N HCl. The culture was maintained at 30°C by using a rotary shaker (150 rpm) for a period of five days before inoculating into a 3-l air-lift fermentor (2.0 l working volume, Hankook Fermentor Co., Korea). Five % (v/v) of the culture was inoculated and cultivated at 25°C with air which was supplied at a rate of 2.5 vvm under batch conditions. The foam was removed by adding 0.05% of anti-foaming agent (Antifoam 289, Sigma, U.S.A.). An initial pH 3.0 was shifted up to 6.0 by adding 1 N NaOH when the mycelial cell growth reached its exponential phase. In order to maintain a constant pH, a pH controller attached to the fermentor was automatically adjusted to the culture broth by adding 5 N NaOH. Then, the system remained stable until the cell growth went into the stationary and/or death phases.

Measurement of Cell Growth, EPS Production and Rheological Parameters

Twenty ml of the sample was collected from the fermentor each day, and then centrifuged at 10,000 ×g for 30 min. The pellet was washed with distilled water and dried at 105°C for 5 h to measure the cell dry weight. Two volumes of acetone was added to the supernatant while whirling with a glass rod. The viscous aggregates wound by the rod were collected as EPS [11]. Harvested EPS were dried at 105°C for 5 h to measure the weight. The viscosity of the culture broth was measured by a Brooksfield viscometer (Synchro-letic type DV-II+, U.S.A.) by collecting 10 ml of the broth from the fermentor where it maintained its constant temperature of 30°C every day. The rheological parameters of the culture broth were estimated by using the following Herschel-Bulkley model [6]:

$$\tau=\tau_y+K\dot{\gamma}^n \quad (1)$$

where τ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (sec^{-1}), τ_y is the yield stress (Pa), K is the consistency index (Pa sec^n) and n(-) is the flow index. The image analysis of the mycelium in the reactor was carried out by an Image Analyzer (Optimas, U.S.A.), fixing the cells by formaldehyde solution with glacial acetic acid [16]. The fractal dimension was calculated by the following equation [2].

$$S_n^{1/2} \propto X_n^{1/D} \quad (2)$$

where S_n is the area of the mycelium (mm^2), X_n is the perimeter (mm) and D is the fractal dimension.

RESULTS AND DISCUSSION

Figure 1 shows the mycelial growth and EPS production in batch cultivation of *G. lucidum* with various concentrations of ammonium phosphate in the medium. It is quite obvious that when the concentrations of ammonium ion is lower than 3 g/l in the medium, it can greatly enhance the cell growth and the cell growth is gradually retarded when the ammonium ion concentrations were increased over the above mentioned concentrations. Maximum cell density measured was 14 g-dry wt/l in ammonium phosphate (3.0 g/l) containing medium after eight days of cultivation, which was about two times higher compared to the ones without any addition of the ammonium ion. However, EPS production was adversely related with the addition of ammonium ion by yielding 7.3 g/l of the maximum EPS production in the absence of ammonium ion. Then, the production gradually decreased as the ammonium ion was increased. In fact, this implies that ammonium ion plays important roles in cell growth and EPS production by affecting the morphology and density of the mycelium during the cultivation process. It is also noticed that the ammonium phosphate has a much more profound effect on mycelial growth than on the EPS production. The change of pH in the medium was not significantly influenced by the concentration of ammonium ion (data not shown).

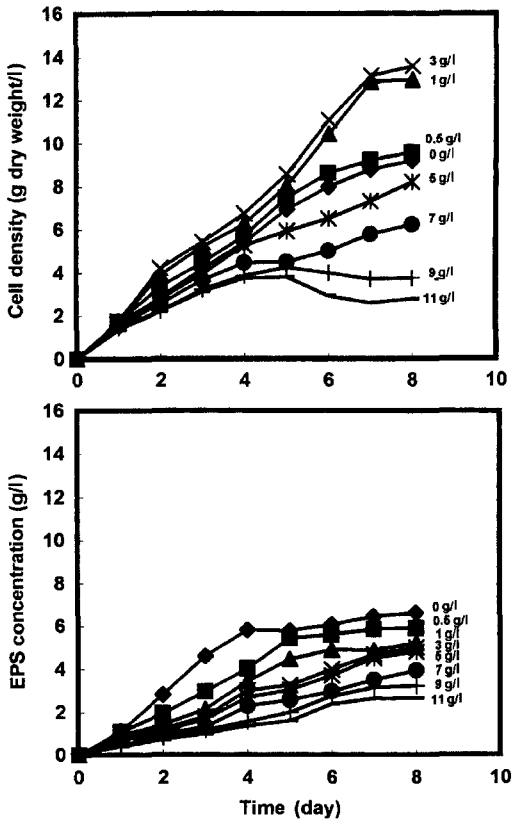

Fig. 1. Kinetics of mycelial growth (MDW) and EPS production for batch cultivation of *G. lucidum* in an air-lift fermenter, adding different concentrations of ammonium phosphates (Initial pH 5).

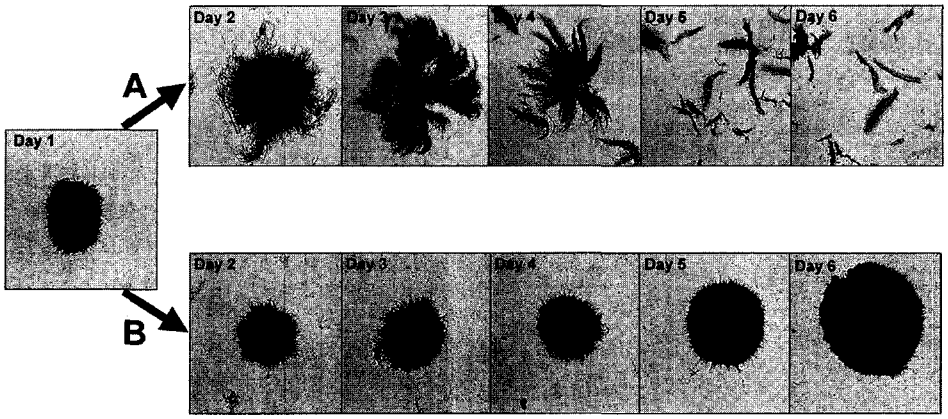

Fig. 2. The morphological changes of *G. lucidum* mycelium in the absence (A) and presence (B) of ammonium phosphate (11 g/l).

Figure 2 also supports the hypothesis on the effect of ammonium ion on cell growth, by showing the change of mycelial structures during the cultivation process. By adding ammonium phosphate, the structure of *G. lucidum* mycelium remained unchanged as pellet forms, which could cause limitation of nutrient transport and this was the reason for the decrease in cell growth. With no or a reduced amount of ammonium phosphate added, the mycelium unfolded, and it seemed to benefit the growth process when it was turned into the filamentous forms. This can explain why better cell growth was observed in a low ammomium phosphate concentration, as shown in Fig. 1. Figs. 3 and 4 show quantitatively the structural changes of the mycelium with various concentrations of ammonium ion for the batch cultivation of *G. lucidum*. In a situation where ammonium ion in the medium was low, higher fractal dimension (1.1-1.25) was estimated, and this implied that the mycelium seemed to be filamentous. On the contrary, when ammonium phosphate concentration was high, a low fractal dimension was calculated, and the mycelium was transformed into pellet forms [17]. A similar pattern was also observed in Fig. 4 showing the changes of the area frequency of the mycelium. In high ammonium phosphate concentrations, the frequency rate of the area in relations to pellets (>1 mm^2) was lower than in

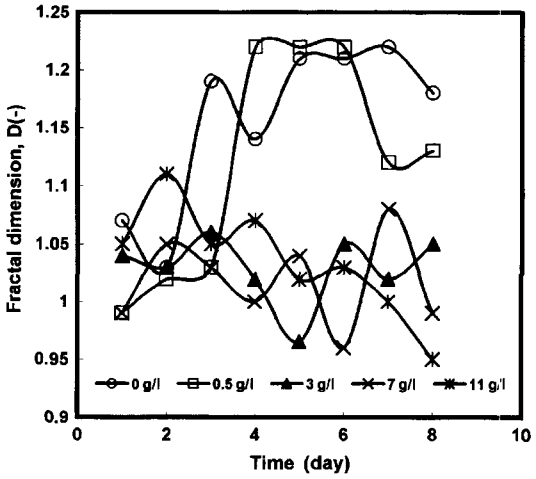

Fig. 3. The change of the fractal dimension of *G. lucidum* by the different cultivations.

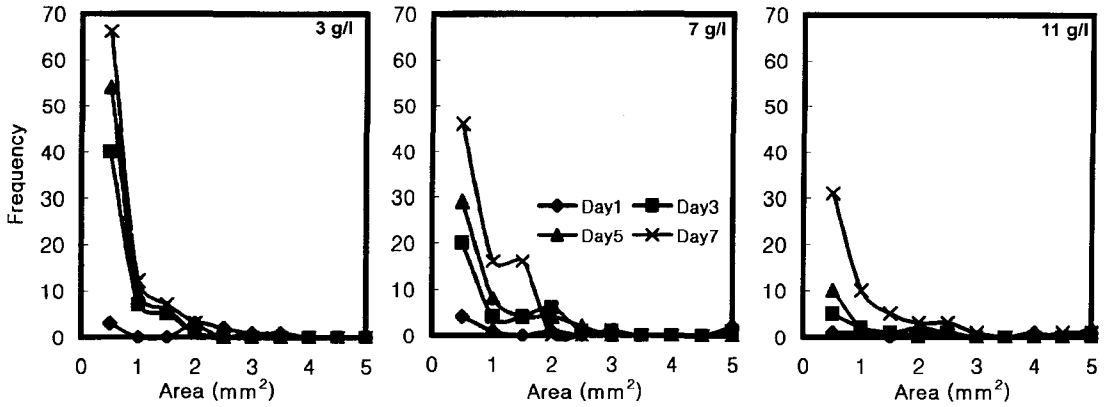

Fig. 4. Variations of the area frequency for pelleted mycelium of *G. lucidum* with different ammonium phosphate concentrations.

low concentrations (<3 g/l). This is another evidence for decrease of mycelial growth in the medium contaning a high concentration of ammonium phosphate. It was also found that the average size of the pellets (>1 mm^2) decreased as the cultivation process continued. Most of the mycelium size in the fermenter fell in the range of 0.5 and 1.5 mm^2, regardless of the cultivation time and ammonium phosphate concentration. In expanding the area of mycelium, there should be several limitations, mostly due to nutrient limitation, increasing shear stress and self-folding activity, etc.

Figures 5 and 6 show the kinds of ammonium phosphate effect on the rheological properties of the filtrate and culture broth during the cultivation. Information derived from these results should be taken seriously in designing and controlling the fermentation process, because it can directly affect the performance of the fermentation system. The shear stresses of both the filtrate and culture broth were increased with a non-linear pattern as the shear rate was increased, and this implied that they had the characteristics of the non-Newtonian fluid [6]. In particular, for the culture broth, a higher shear stress was observed in the absence of ammonium phosphate at the same shear rate as compared with those in the presence of ammonium phosphate. This was due to the high mycelial growth and filamentous structure. The shear stress was increased in the latter phase of the cultivation for all the cases solely because of the morphological change of the mycelium in addition to the increase of the cell density. However, regardless of ammonium phosphate concentration, the shear diagrams of the culture filtrate were much less affected compared to the culture broth. This means that the rheological properties of the culture broth is significantly influenced by mycelial concentration, but not by EPS concentration. It also confirmed that the changes of the morphology of the mycelium are crucial in the growth of mycelium and in decreasing EPS in *G. lucidum*. The data in Figs. 5 and 6 were well fitted to the Herschel-Bukley model ($R^2 \geq 0.95$) [6]. Model parameters of consistency index, K, and flow index, n, were estimated and the results are shown in Table 1. Regardless of the existence of ammonium phosphate, values of K and yield stress for both the culture broth and filtrate increased with the increase of fermentation time, while n values decreased.

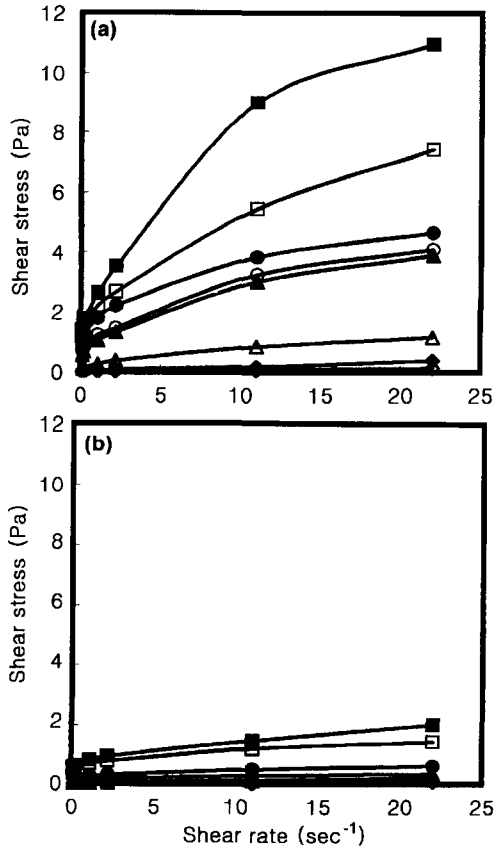

Fig. 5. Flow curves for culture broth (a) and culture filtrate (b) of *G. lucidum* cultured in the absence of ammonium phosphate at initial pH 5.
◇ Day 1; ◆ Day 2; △ Day 3; ▲ Day 4; ○ Day 5; ● Day 6; □ Day 7; ■ Day 8.

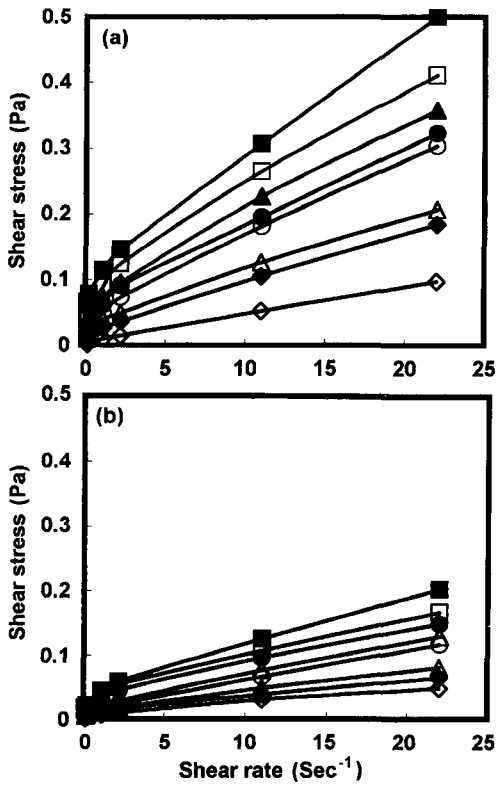

Fig. 6. Flow curves for culture broth (a) and culture filtrate (b) of *G. lucidum* cultured in the presence of ammonium phosphate (7 g/l) at initial pH 5.
◇ Day 1; ◆ Day 2; △ Day 3; ▲ Day 4; ○ Day 5; ● Day 6; □ Day 7; ■ Day 8.

Table 1. Rheological parameters of culture broth and culture filtrate of *G. lucidum* cultured in the presence and the absence of ammonium phosphate during batch cultivation.

Concentration of NH_4^+ (g/l)	Time (Day)	Culture broth			Culture filtrate		
		Consistency index K (PaS^n)	Flow index n(-)	Yield stress τ_y (Pa)	Consistency index K (PaS^n)	Flow index n(-)	Yield stress τ_y (Pa)
0 (g/l)	1	0.31	0.93	-	0.28	0.89	-
	2	0.62	0.83	0.2	0.32	0.82	-
	3	1.59	0.47	0.64	0.61	0.83	0.11
	4	2.90	0.30	1.20	1.21	0.74	0.15
	5	3.10	0.33	0.98	1.52	0.25	0.15
	6	3.55	0.29	1.68	1.55	0.23	0.12
	7	3.98	0.33	1.86	2.27	0.23	0.32
	8	4.40	0.40	3.3	2.50	0.24	0.34
1 (g/l)	1	0.03	0.88	-	0.07	0.91	-
	2	0.08	0.67	0.02	0.11	0.83	-
	3	1.24	0.49	0.23	0.33	0.51	0.10
	4	1.46	0.50	0.30	0.34	0.53	0.11
	5	2.64	0.64	0.49	0.35	0.53	0.13
	6	2.46	0.61	0.44	0.34	0.58	0.15
	7	2.40	0.60	0.35	0.39	0.53	0.15
	8	2.40	0.58	0.24	0.43	0.55	0.20
7 (g/l)	1	0.33	0.90	-	0.27	0.93	-
	2	0.48	0.83	0.01	0.33	0.86	0.04
	3	0.54	0.8	0.06	0.36	0.84	0.03
	4	0.72	0.73	0.22	0.44	0.80	0.03
	5	0.65	0.74	0.06	0.40	0.81	0.02
	6	0.70	0.72	0.09	0.52	0.75	0.03
	7	0.79	0.71	0.09	0.55	0.74	0.07
	8	0.81	0.72	0.17	0.58	0.74	0.11

Thus, the culture broth and filtrate of *G. lucidum* exhibited the pseudoplastic behavior with yield stress. Variations of K and n values of culture broth corresponding to fermentation time were much higher than those of culture filtrate. This implies that the flow behavior of the culture fluid can mainly be influenced by mycelial concentration, as shown in Figs. 5 and 6. However, in a case of where ammonium phosphate was zero or low (1%), larger K and smaller n values were mostly estimated compared to those in higher ammonium ion concentration (7%). Since the filamentous and pelleted growth were observed in the absence and presence of ammonium phosphate, respectively, these results suggested that the change of flow behavior during the batch culture was due to mycelial morphology

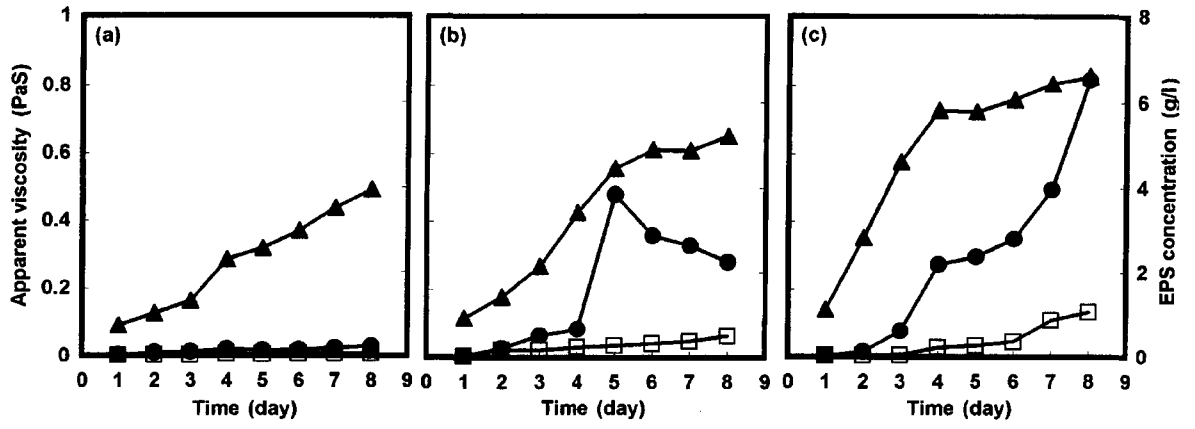

Fig. 7. Time profiles of EPS production (—▲—) and apparent viscosity on the culture broth (—●—) and culture filtrate (□) of *G. lucidum* cultured in the presence and the absence of ammonium phosphate.
(a) 7 g/l ammonium phosphate; (b) 1 g/l ammonium phosphate; (c) without ammonium phosphate.

as well as mycelial concentration. These results were similar to the results observed with other mycelial growth [9].

Figure 7 also demonstrates the effect of ammonium ion on the viscosity change during the fermentation. In high ammonium phosphate concentration, the apparent viscosity of the culture broth and filtrate did not appear to change much by the cultivation time, because of minimum levels of the mycelial growth and EPS production. The continuous increase of the culture filtrate viscosity was observed without adding ammonium phosphate while yielding maximum EPS production, and high cell growth was observed by a large increase of the broth viscosity. In 1 g/l of ammonium phosphate, the apparent viscosity of the culture broth dropped during the fermentation, and the main reason for this fall appeared to be cell lysis. Continuous EPS production (also represented by an increase of apparent viscosity of the culture filtrate) was observed in 1 g/l of ammonium phosphate or in the absence of ammonium ion, although the mycelial growth (expressed as culture broth) was decreased. On the contrary, the EPS production level was not high in the culture when the cells were grown in high ammonium phosphate concentration as compared with that without ammonium phosphate. This suggests that an appropriate amount of ammonium phosphate should be added in the medium, depending on the purpose of fermentation, such as for high cell growth or high EPS production.

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REFERENCES

1. Aharonowitz, Y. 1980. Nitrogen metabolite regulation of antibiotic biosynthesis. *Annu. Rev. Microbiol.* **34:** 209-233.
2. Aratani, T., T. Fujii, T. Morikawa, and K. Miyanani. 1988. Evaluation of floc form by fractal dimension. *J. Chem. Eng.* **14:** 395-400.
3. Braun, S. and S. E. Vecht-Lifshiz. 1991. Mycelial morphology and metabolite production. *Trends in Biotechnol.* **9:** 63-68.
4. Bulmer, M. A., B. J. Catley, and P. J. Kelly. 1987. The effect of ammonium ions and pH on the elaboration of the fugal extracellular polysaccharide, pullulan, by *Aureobasidium pullulans*. *Appl. Microbiol. Biotechnol.* **25:** 362-365.
5. Cavazzoni, V. and A. Adami. 1992. Exopolysaccharides produced by mycelial edible mushrooms. *Ital. J. Food Sci.* **1:** 9-15.
6. Holdsworth, S. D. 1971. Applicability of rheological models to the interpretation of flow and processing behavior of fluid food products. *J. Texture Studies* **2:** 393-418.
7. Jong, S. C. and J. M. Birmingham. 1992. Medicinal benefits of the mushroom *Ganaderma*. *Advances in Appl. Microbiol.* **37:** 101-134.
8. Kechang, Z., W. Xing, X. Rou, and Z. Tao. 1994. Studies of submerged fermentation of *G. lucidum* and crude polysaccharide of *G. lucidum* sp. pp. 119-121. *In* W. K. Teo, M. S. G. Yap, and S. K. Oh (eds.), *Better Living through Innovative Biological Engineering*. Continental Press. Singapore.
9. Kim, J. H., J. M. Lebealt, and M. Reuss. 1983. Comparative study on rheological properties of mycelial broth in filamentous and pelleted forms. *Eur. J. Appl. Microbiol. Biotechnol.* **18:** 11-16.
10. Lee, S. Y. and T. S. Kang. 1997. Optimization of antitumor active exo-polysaccharide production through the submerged cultivation of *G. lucidum* mycelium. *Kor. J. Biotechnol. Bioeng.* **25:** 139-145.
11. Lee, S. Y. and T. S. Kang. 1996. Production conditions and characterization of the exo-polymer produced by submerged cultivation of *G. lucidum* mycelium. *Kor. J. Appl. Biotechnol. Bioeng.* **24:** 111-118.
12. Maiero, R. and V. L. Bononi. 1995. Exopolysaccharides and biomass production in submerged culture by edible mushrooms. pp. 887-892. *In* T. J. Ellitt (ed.). *Science and Cultivation of Edible Fungi*, Vol. 2, A. Balkema Press, Rotterdam, Netherlands.
13. Metz, B., E. W. Bruijn, and J. C. van Suijdam. 1981. Method for quantitative representation of morphology of molds. *Biotechnol. Bioeng.* **23:** 149-162.
14. Mizuno, S. 1988. Development and utilization of bioactive substances from mushroom fungi. *Food Processing.* **23:** 37-43.
15. Pace, G. W. 1980. Rheology of mycelial fermentation broth. pp. 95-110. *In* J. E. Smith, D. R. Berry, and B. Kristiansen (eds.). *Fungal Biotechnology.* Academic Press, London, U.K.
16. Packer, H. L. and C. R. Thomas. 1990. Morphological measurements on filamentous microorganisms by fully automatic image analysis. *Biotechnol. Bioeng.* **35:** 870-881.
17. Patankar, D. B., T. Liu, and T. Oolman. 1993. A fractal model for the characterization of mycelial morphology. *Biotechnol. Bioeng.* **42:** 571-578.
18. Sone, Y., R. Okuda, A. Wada, A. Kishida, and A. Misaki. 1985. Structures and antitumor activities of the polysaccharides isolated from fruiting body and the growing culture of mycelium. *Agr. Biol. Chem.* **49:** 2642-2650.
19. Suijdam. J. C. V. and B. Metz. 1981. Influence of engineering variables upon the morphology of filamentous molds. *Biotechnol. Bioeng.* **23:** 111-148.
20. Tseng, T. C. 1984. Studies on *Ganoderma lucidum* 1. Liquid culture and chemical composition of mycelium. *Bot. Bull. Axad. Sin. (Taipei)* **25:** 149-154.