Biotechnology and Bioprocess Engineering 15: 453-459 (2010)
DOI 10.1007/s12257-008-0265-3

RESEARCH PAPER

Effects of Ascorbic Acid and Uracil on Exo-polysaccharide Production with *Hericium erinaceus* in Liquid Culture

Jong Seok Lee, Jae Wan Wee, Hye Young Lee, Hyo Sil An, and Eock Kee Hong

Received: 8 November 2008 / Revised: 28 October 2009 / Accepted: 13 November 2009

Abstract *Hericium erinaceus* is a well known edible and medicinal mushroom used in East-Asia. Recently, *H. erinaceus* has attracted a lot of attention owing to its anti-tumor, immuno-modulatory, and cytotoxic effect. It has been postulated that the fruiting body of *H. erinaceus* contains a polysaccharide that is similar to β-D-glucan, which is known to have antitumor activity against Sarcoma 180. However, optimized liquid culture conditions for enhanced polysaccharide productivity have yet to be developed, which is a necessary step for industrial applications. Therefore, the aim of this study was to determine the optimal liquid culture conditions for maximum polysaccharide production. In shake flask cultures, the optimal concentration of ascorbic acid was found to be 2.0 g/L, which prevented the broth from changing color from yellow to black. The optimal culture conditions were determined to be 23°C, 200 rpm, and a 10% inoculum size, at an uncontrolled initial pH. In addition, the modified medium contained 20 g/L glucose, 10 g/L yeast extract, and 2.0 g/L ascorbic acid. The maximum mycelial biomass and exo-polysaccharide (EPS) production in the modified medium containing uracil was 13.43 and 1.26 g/L, respectively.

Keywords: *Hericium erinaceus*, exo-polysaccharide, ascorbic acid, uracil, mycelial morphology

Jong Seok Lee, Jae Wan Wee, Hye Young Lee, Hyo Sil An,
Eock Kee Hong*
Department of Bioengineering and Technology, Kangwon National University, Chuncheon 200-701, Korea
Tel: +82-33-250-6275; Fax: +82-33-243-6350
E-mail: ekhong@kangwon.ac.kr

1. Introduction

Hericium erinaceus belongs to the Aphyllophorales, Hydnaecae, and Hericium families and is a well known edible and medicinal mushroom in Japan and China. *H. erinaceus* has been reported to have various biological activities such as anti-microbial effect [1,2], anti-tumor activities [3-5], immunomodulatory effect [6], antioxidant properties [7,8], cytotoxic effect [9,10], hypolipidemic effect [11], and promoting the synthesis of the neurogrowth factor [12,13]. In addition, this fungus has been used for treating and curing gastric ulcers, gastroduodenal ulcers, and chronic gastricism, among other ailments [14]. It has been reported that *H. erinaceus* contains various constituents including erinacine, hericenone, terpenoids, proteins, lipids, and polysaccharides. Since the polysaccharides from mushrooms are known to have no toxic side effects, unlike the existing anti-cancer chemical medications, treatment using polysaccharide-based therapies has shown very promising results in prolonging the life span of a patient [15,16]. The mycelial morphology of fungus is an important parameter that affects the physical properties of the culture broth. Rheological behavior is intimately associated with morphology and biomass concentration [17,18]. It was reported that medium constituents and culture pH affected morphological changes in mycelia [19].

In the present study, liquid culture conditions for exo-polysaccharide (EPS) production by *H. erinaceus* were optimized using a submerged culture, which has the potential for higher metabolite production in a compact space and over a shorter time span with less chance of contamination.

2. Materials and Methods

2.1. Strain and media

The mycelia were isolated from the fruiting body of *H. erinaceus*, the higher basidiomycete's mushroom, which was collected from Gumi, Gyeongsangbuk-do, Korea. They were cultured on an agar plate containing potato dextrose agar (PDA) that was supplemented with antibiotics (50 μg/mL of streptomycin and 60 μg/mL of ampicillin). The PDA contained 10 g/L potato, 50 g/L glucose, 5.0 g/L peptone, 5.0 g/L yeast extract, 1.5 g/L K_2HPO_4, 0.5 g/L $MnSO_4$, and 15 g/L agar. The basal medium for mycelial growth was YMK, which contained 20 g/L glucose, 5.0 g/L yeast extract, 2.0 g/L KH_2PO_4, and 1.0 g/L $MgSO_4 \cdot 7H_2O$.

2.2. Flask cultures

The seed culture was cultivated for 4 days in 100 mL of the YMK media in a 250 mL flask that was inoculated with 10 mL of an activated stock solution frozen at −70°C. For mycelial growth, the mycelia were homogenized with a Heidolph DIAX 600 homogenizer (VWR International, West Chester PA, USA), and 10% of the seed culture broth was inoculated into 100 mL of the GY media, which contained 20 g/L glucose and 10 g/L yeast extract, in a 250 mL flask. They were then cultivated for 7 days at 23°C with 200 rpm in a shaking incubator (Vision Scientific Co., Ltd., Buchun, Korea).

2.3. Analytical methods

After 7 days of cultivation, the culture broth was centrifuged at 5,000 rpm for 20 min. Precipitated mycelia were washed three times with distilled water and then dried for 24 h at 60°C. The EPS derived from the liquid culture broth was prepared by ethanol precipitation with three times volume followed by standing at 4°C overnight, filtered with 0.45 μm Whatman filter paper, and then dried in a drying oven to a constant weight. The dry weight of mycelia and EPS was quantified by subtracting the dry weight of the filter paper from the total weight. The residual glucose in cultured broth was determined using a glucose assay kit (Sigma Diagnostics St. Louis, MO, USA) and glucose analyzer (YSI Inc., Yellow Springs, Ohio, USA) according to the manufacturer's instructions.

3. Results and Discussion

3.1. Effects of ascorbic acid on EPS production

When *H. erinaceus* were grown in liquid culture, the culture broth appeared dark and gave rise to poor mycelial growth at the end of the culture period [20]. The yeast-like fungus *Aureobasidium pullulans* is known to produce pullulan, which in turn typically produces a black melanin pigment. For industrial production of pullulan, color variant strains, which are brightly pigmented, are used to produce pullulan with less contaminating pigment; however, these variants have less productivity [21]. In the present study, we investigated the effects of ascorbic acid on mycelial growth and EPS production with the aim of protecting the culture broth from melanism. Among the different ascorbic acid concentration that we examined, 2.0 g/L of ascorbic acid was found to effectively eliminate

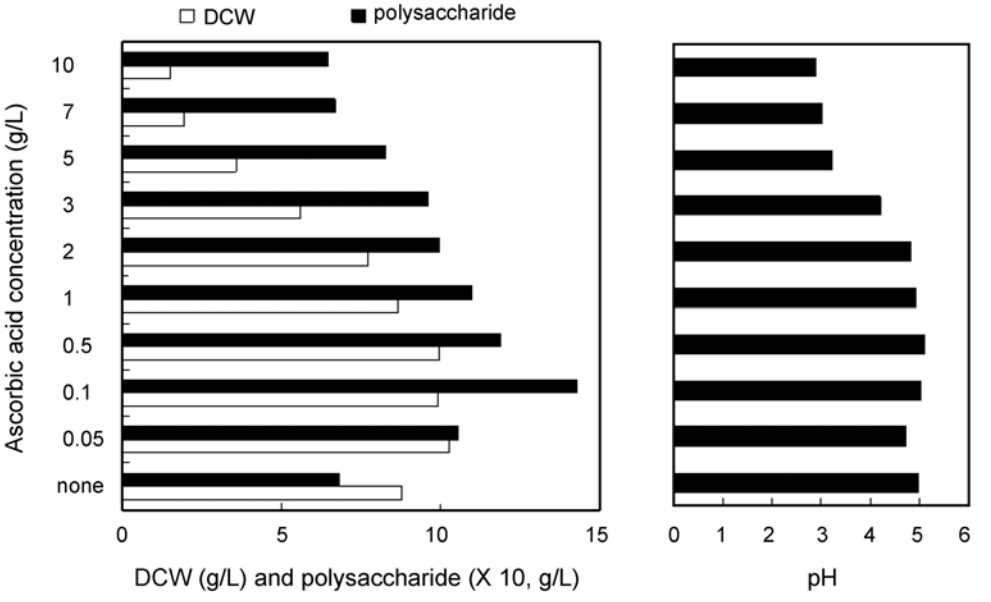
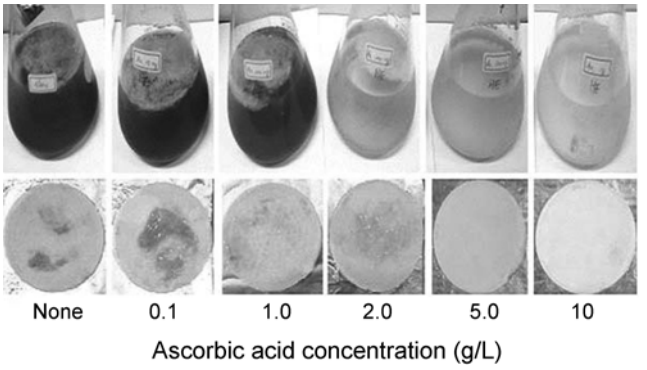


Fig. 1. The effect of ascorbic acid on mycelial growth and EPS production in GY medium. The mycelia were cultivated for 7 days at 23°C with 200 rpm, uncontrolled pH, and a 10 % inoculum size.



S. 1. The effect of ascorbic acid on the color of the liquid culture broth (up) and EPS (down) in GY medium.

melanism in the culture broth (Fig. 1 and S. 1).

3.2. Effect of initial pH on the mycelial morphology, growth, and EPS production

To investigate the effect of initial pH on mycelial growth and EPS production, *H. erinaceus* were cultivated in the GYA medium under the different initial pH (3.0 ~ 8.0). Initial pH was controlled using 1N NaOH and 1N HCl. The pH value of the non-control group was 4.50. As a result of the pH profiles during culture, when the initial pH ranged from 3.0 to 5.0, the pH increased. In contrast, when the initial pH ranged from 6.0 to 8.0, the pH decreased towards 5.0. From these experiments, mycelial growth of *H. erinaceus* was found to be maximal at pH 5.0 (Fig. 2). It has been shown that mycelia produce organic acids, which decrease the medium pH to optimal culture conditions. In addition, it is known that mycelial growth in most basidiomycetes is higher in low medium pH. The optimum pH for mycelial growth in *Lentinus edodes*, *Coriolus versicolor*, and *Tricholoma matsutake* was 4.5, 5.5, and 5.0, respectively [22]. It was reported that the optimum pH for mycelial growth in *H. erinaceus* in solid culture was 4.0 and mycelial growth in *H. erinaceus* was sensitive of pH [23]. Among the different initial pH values examined, the highest EPS production (0.994 g/L) was obtained in the non-control group (initial pH 4.5). The second highest EPS production occurred when the initial pH ranged from 7.0 to 8.0 as the culture pH shifted to acidic and neutral ranges (Fig. 2). The optimum pH values for EPS production in *Schizophyllum commune* and *A. pullulans* have been reported to be 6.0 and 4.0, respectively [24,25]. The EPS production is frequently dependent on a desirable mycelial morphology for most fungal fermentation [33,34]. In Fig. 3, the mycelial morphology during mycelial development in the initial pH 3 group (poor mycelial growth and EPS production) and non-control initial pH group (pH 4.5, higher mycelial growth and EPS production) were examined by high-resolution polarizing microscope (Carl Zeiss Co., Germany). Long and branched filamentous

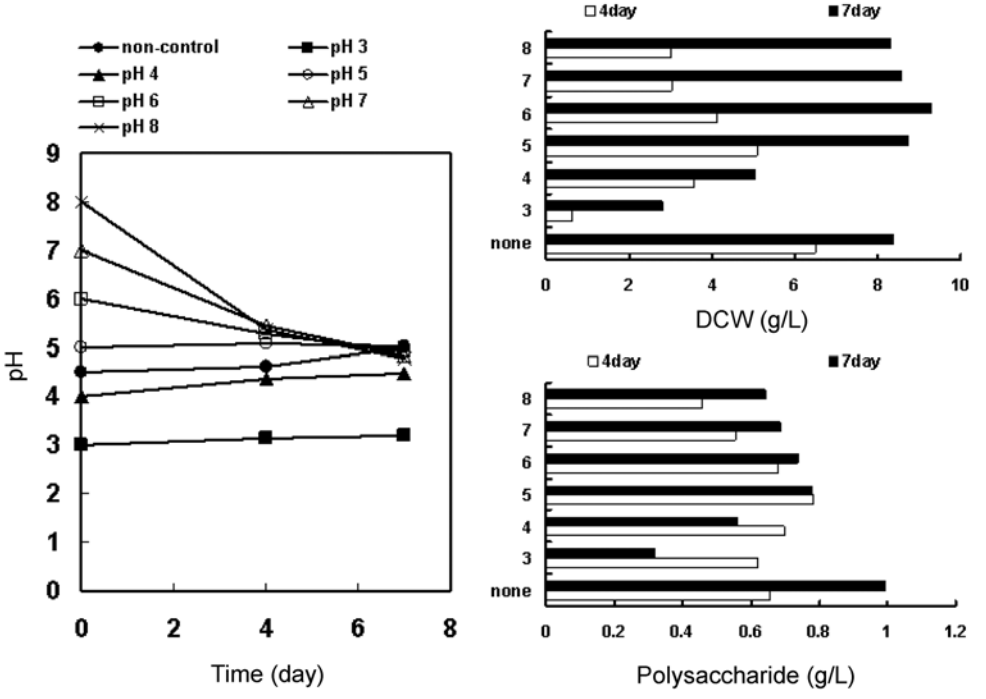


Fig. 2. The effects of initial pH on mycelial growth and EPS production in GYA medium. The mycelia were cultivated at 23°C with 200 rpm and a 10% inoculum size. The initial pH values were controlled using 1N NaOH and 1N HCl. The pH value of the non-control group was 4.50.

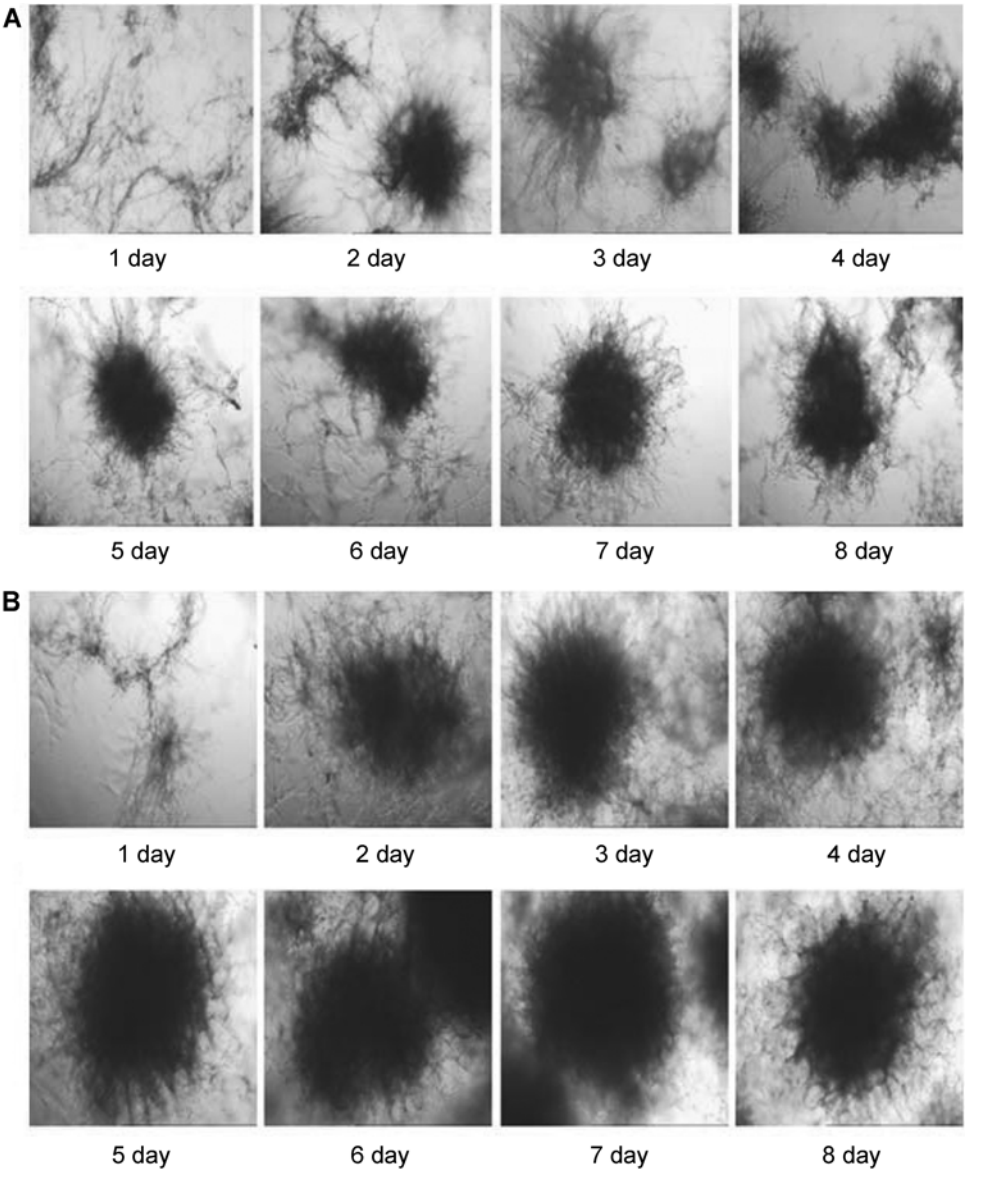


Fig. 3. Images of mycelial morphology of *H. erinaceus* at various time points in a shake flask culture. (A) Mycelial morphology of *H. erinaceus* at initial pH 3. (B) Mycelial morphology of *H. erinaceus* at non-controlled initial pH. The mycelia were cultivated at 23°C with 200 rpm and a 10% inoculum size. Mycelial morphology was analyzed by an image analysis system (Carl Zeiss Co., Germany). One milliliter of mycelium was randomly sampled and was fixed with 1 mL of a 3-fold diluted solution of fixation solution (13 mL of 40% formaldehyde + 5 mL of glacial acetic acid + 200 mL of 50% EtOH).

mycelia were observed in both the initial pH 3 and non-control group at an early stage of the culture. Meanwhile, at a later stage of the culture, clumped and entangled mycelium were observed in the initial pH 3 group and short branched and entangled mycelium, in addition to a rough pellet, were observed in the non-control initial pH group. *A. pullulans* are known to change to a black color as a result of chlamydospore production and the ratio of yeast-like cells is affected by culture pH. The culture pH, within a certain range, affected not only the cell morphology, but also the pullulan productivity of *A. pullulans* [27]. *Rhizopus arrhizus* showed clumped and pellet growth at 10 ~ 20 g/L peptone [28]. Filamentous mycelia were observed at low ammonium ion concentrations, but pellets were observed at high concentration when *Ganoderma lucidium* were grown in liquid culture [29].

3.3. Effect of carbon source and C/N ratio

To maximize mycelial growth of *H. erinaceus*, we tested the following carbon sources; salicin, lactose, glycerol, D-ribose, D-mannose, arabinose, cellobiose, D-maltose, D-

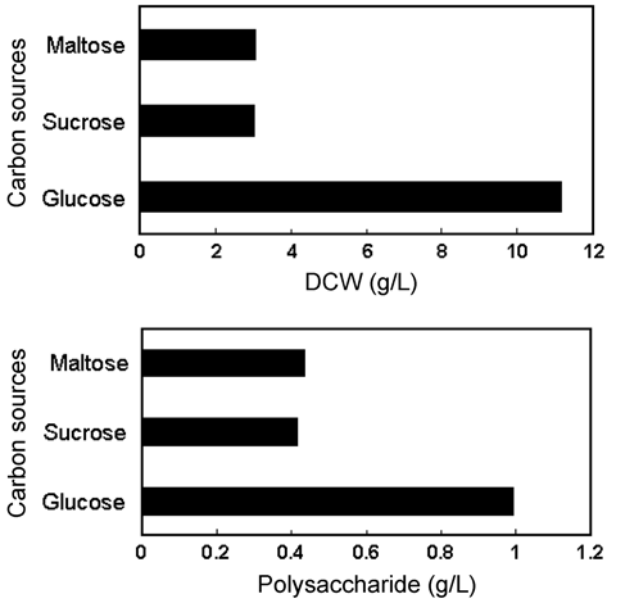


Fig. 4. The effects of different carbon sources on mycelial growth and EPS production in GYA medium. The mycelia were cultivated for 7 days at 23°C with 200 rpm, pH 4.5, and a 10% inoculum size. Glucose in the GYA medium was replaced by various other carbon sources. The final concentration of the carbon source was 2.0% (w/v).

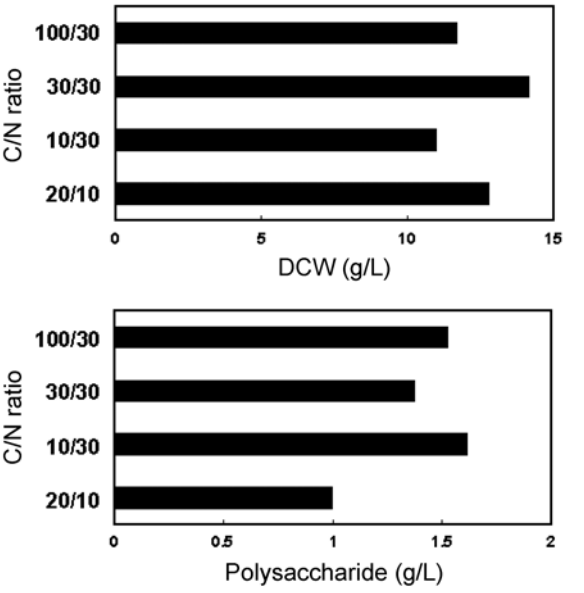


Fig. 5. The effects of the C/N ratio on mycelial growth and EPS production. The mycelia were cultivated for 7 days at 23°C with 200 rpm, pH 4.5, and a 10% inoculum size.

xylose, adonitol, D-fructose, D-galactose, D-glucose, mannitol, sucrose, D-sorbitol, dextrin, and starch [23]. The highest level of EPS production of *H. erinaceus* was observed in media that contained sucrose followed by maltose, and glucose [20]. In contrast with a previous report, the highest mycelial growth (11.1 g/L) and EPS production (0.994 g/L) of *H. erinaceus* was obtained in the media containing glucose (Fig. 4).

To examine the effects of the carbon-to-nitrogen ratio (C/N ratio) on EPS production, the concentration of glucose in the medium was varied from 10 to 100 g/L. As shown in Fig. 5, a lower concentration of glucose was preferred for mycelial growth. At higher glucose concentrations, substrate availability for mycelial growth and EPS production decreased. It is believed that catabolite repression is a general phenomenon in metabolite production when using a microorganism [30]. In addition, it is generally known that an increased accumulation of bioactive polymers and a decreased availability of the nitrogen source occur at a higher C/N ratio [31].

3.4. Effect of uracil on EPS production

UDP-glucose, which is used as a precursor of polymer synthesis, is an essential factor in the EPS production rate. In addition, it is known that uracil, which is a precursor of UDP-glucose, and its kinase is key factors in polymer synthesis. To investigate the effects of uracil on mycelial growth and EPS production, the concentration of uracil in medium that was adjusted to an initial pH of 4.5 was varied from 0.1 to 1.0 g/L. On the 4th day of liquid culture, the highest EPS production was obtained in medium containing 0.3 g/L uracil. On the 7th day of liquid culture, the highest EPS production was achieved in media that was supplemented with 1.0 g/L uracil (Fig. 6). It has been postulated that uracil acts as upregulation effectors of polymer synthesis at the expense of the nitrogen source. To confirm this, the effect of uracil addition on the 4th day of liquid culture on EPS production was examined. In these experiments, the concentration of uracil in the medium was varied from 0.1 to 1.0 g/L. Overall, mycelial growth and EPS production in media that was supplemented with uracil on the 4th day of liquid culture was significantly higher than in media that was not supplemented with uracil (Fig. 7). Similar results were observed in a previous study, where uracil was used as a precursor of polymer synthesis in the *Agrobacterium* sp and the concentration of UMP and AMP correspondingly increased at the expense of the nitrogen source [32].

4. Conclusion

In this study, the optimal medium composition of the liquid culture for *H. erinaceus* was investigated with the aim of maximizing EPS production and mycelial growth. From these experiments we determined that the optimal medium contained 20 g/L glucose, 10 g/L yeast extract, and 2.0 g/L

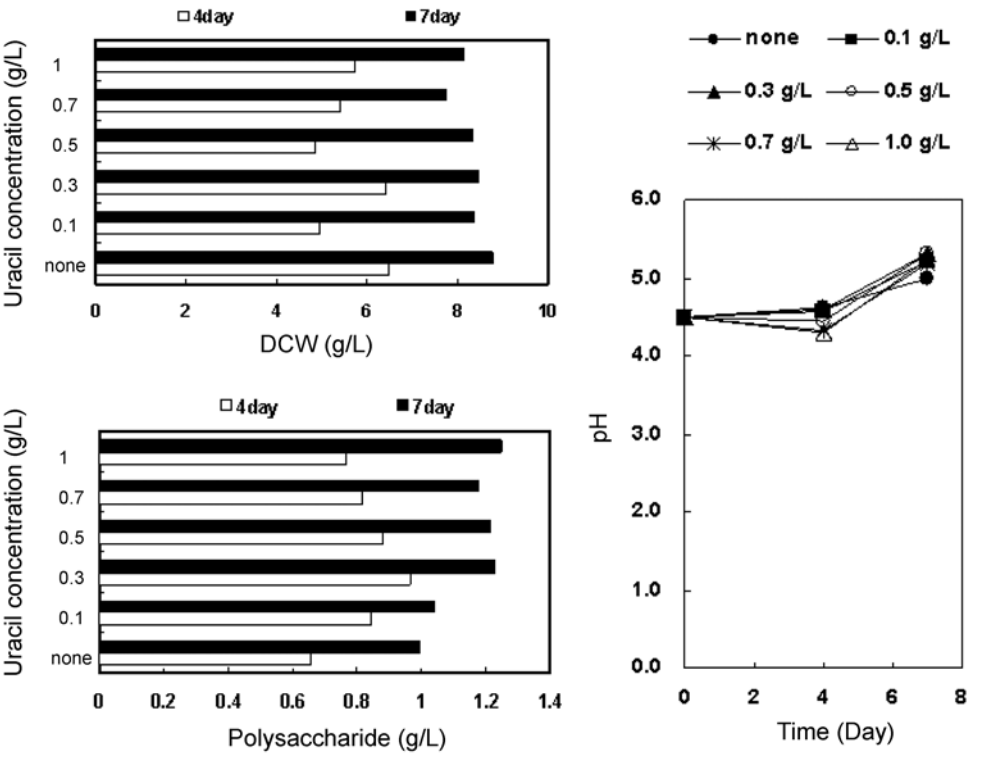


Fig. 6. The effects of uracil concentration on mycelial growth and EPS production in GYA medium. The mycelia were cultivated at 23°C with 200 rpm, pH 4.5, and a 10% inoculum size.

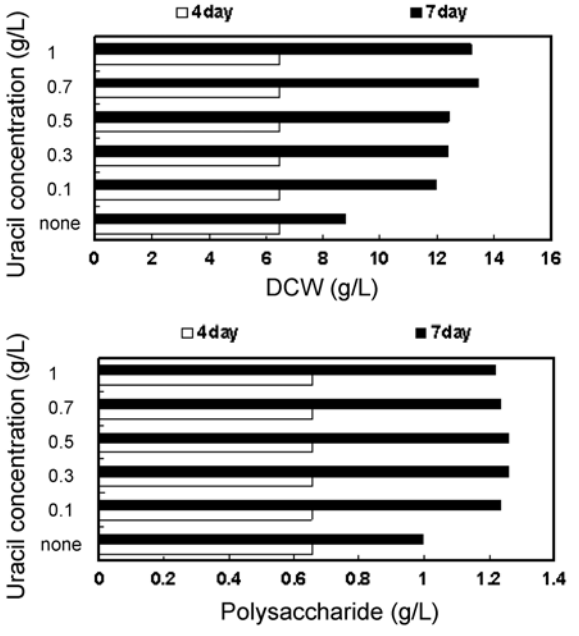


Fig. 7. The effects of uracil addition on the 4th day of liquid culture on mycelial growth and EPS production in GYA medium. The mycelia were cultivated at 23°C with 200 rpm, pH 4.5, and a 10% inoculum size. The uracil concentration in the medium was varied from 0.1 to 1.0 g/L.

ascorbic acid. In shake flask, the maximum mycelial growth and EPS production in media that was supplemented with 0.7 and 0.3 g/L uracil on the 4th day of liquid culture were 13.43 and 1.26 g/L, respectively.

References

1. Okamoto, K., A. Shimada, R. Shirai, H. Sakamoto, S. Toshida, F. Ojima, Y. Ishiguro, T. Sakai, and H. Kawagishi (1993) Antimicrobial chlorinated orcinol derivates from mycelia of *Hericium erinaceum*. *Phytochem*. 34: 1445-1446.
2. Kim, D. M., C. W. Pyun, H. G. Ko, and W. M. Park (2000) Isolation of antimicrobial substances from *Hericium erinaceum*. *Mycobiol*. 28: 33-38.
3. Mizuno, T., T. Wasa, H. Ito, C. Suzuki, and N. Ukai (1992) Antitumor active polysaccharides isolated from the fruiting body of *Hericium erinaceus*, an edible and medicinal mushroom called yamabushitake or houtou. *Biosci. Biotechnol. Biochem*. 56: 347-348.
4. Mizuno, T., H. Saito, T. Nishitoba, and H. Kawagishi (1995) Antitumor active substances from mushrooms. *Food Rev. Int*. 11: 23-61.
5. Kwon, S. H., C. N. Kim, C. Y. Kim, S. T. Kwon, K. M. Park, and S. Hwangbo (2003) Antitumor activities of protein-bound polysaccharide extracted from mycelia of mushroom. *Kor. J. Food Nutr*. 16: 15-21.
6. Liu, C. P., J. N. Fang, X. Y. Li, and X. Q. Xiao (2002) Structural characterization and biological activities of SC4, an acidic polysaccharide from *Salvia chinensis*. *Acta Pharmacol. Sin*. 23: 162-166.
7. Park, S. S., K. H. Yu, and T. J. Min (1998) Antioxidant activities of extracts from fruiting bodies of mushrooms. *Kor. J. Mycol*.

26: 69-77.
8. Mau, J. L., H. C. Lin, and S. F. Song (2002) Antioxidant properties of several specialty mushrooms. *Food Res. Int.* 35: 519-526.
9. Kawagishi, H., M. Ando, and T. Mizuno (1990) Hericenone A and B as cytotoxic principles from the mushroom *Hericium erinaceum*. *Tetrahed. Lett.* 31: 373-376.
10. Kuwahara, S., E. Morihiro, A. Nemoto, and A. Hiramatsu (1992) Synthesis and absolute configuration of a cytotoxic fatty acid isolated from the mushroom, *Hericium erinaceum*. *Biosci. Biotechnol. Biochem*. 56: 1417-1419.
11. Yang, B. K., J. B. Park, and C. H. Song (2002) Hypolipidemic effect of exo-polymer produced in submerged mycelial culture of five different mushrooms. *J. Microbiol. Biotechnol.* 12: 957-961.
12. Kawagishi, H., A. Shimada, R. Shirai, K. Okamoto, F. Ojima, H. Sakamoro, Y. Ishiguro, and S. Furukawa (1994) Erinacines A, B and C, strong stimulators of nerve growth factor (NGF) synthesis from the mycelia of *Hericium erinaceum*. *Tetrahed. Lett*. 35: 1569-1572.
13. Lee, E. W., K. Shizuki, S. Hosokawa, M. Suzuki, H. Suganuma, T. Inakuma, J. Li, M. Ohnishi-Kameyama, T. Nagata, S. Furukawa, and H. Kawagishi (2000) Two novel diterpenoids, erinacines H and I from the mycelia of *Hericium erinaceum*. *Biosci. Biotechnol. Biochem*. 64: 2402-2405.
14. Lu, L., J. Li, and Y. Cang (2002) PCR-based sensitive detection of medicinal fungi *Hericium* species from ribosomal internal transcribed spacer (ITS) sequences. *Biol. Pharm. Bull*. 25: 975-980.
15. Komatsu, N., S. Okubo, S. Kikumoto, K. Kimura, and G. Saito (1969) Host-mediated antitumor action of schizophyllan, a glucan. Produced by *Schizophyllum commune*. *Gann*. 60: 137-144.
16. Wang, Z., D. Luo, and Z. Liang (2004) Structure of polysaccharides from the fruiting body of *Hericium erinaceus Pers*. *Carbohydr. Polymer*. 57: 241-247.
17. Braun, S. and S. E. Vecht-Lifshitz (1991) Mycelial morphology and metabolite production. *Trends Biotechnol*. 9: 63-68.
18. Reols, A. J., V. Berg, and R. M. Voncken (1974) Rheology of mycelial broths. *Biotechnol. Bioeng*. 16: 181-208.
19. Shin, Y. C. and S. M. Byun (1991) Effects of pH on the elaboration of pullulan and the morphology of *Aureobasidium pullulans*. *Kor. J. Appl. Microbiol. Biotechnol*. 19: 193-199.
20. Seo, J. S. (2003) *Study on the process development for cell growth with Hericium erinaceus in liquid cultivation*. M.S. Thesis. Kangwon National University, Korea.
21. Leathers, T. D., G. W. Nofsinger, C. P. Kurtzman, and R. J. Bothast (1988) Pullulan production by color variant strains of *Aureobasidium pullulans*. *J. Ind. Microbiol*. 3: 231-239.
22. Park, K. H. and J. S. Lee (1991) Optimization of media composition and culture conditions for the mycelia growth of *Coriolus versicolor* and *Lentinus edodes*. *Kor. J. Biotechnol. Bioeng*. 6: 91-98.
23. Ko, H. G., D. M. Kim, and W. M. Park (1997) Composition of a new medium for mycelial growth of *Hericium erinaceus*. *Kor. J. Mycol*. 25: 369-376.
24. Kumari, M., S. A. Survase, and R. S. Singhal (2008) Production of schizophyllan using Schizophyllum commune NRCM. *Bioresour. Technol*. 99: 1036-1043.
25. Auer, D. P. and R. J. Seviour (1990) Influence of varying nitrogen sources on polysaccharide production by *Aureobasidium pullulans* in batch culture. *Appl. Microbiol. Biotechnol*. 32: 637-644.
26. Gupta, K., P. K. Mishra, and P. Srivastava (2007) A correlative evaluation of morphology and rheology of *Aspergillus terreus* during lovastatin fermentation. *Biotechnol. Bioproc. Eng*. 12: 140-146.
27. Heald, P. J. and B. Kristiansen (1985) Synthesis of polysaccharide by yeast-like forms of *Aureobasidium pullulans*. *Biotechnol. Bioeng*. 27: 1516-1519.
28. Byrne, G. S. and O. P. Ward (1989) Effect of nutrition on pellet by *Rhizopus arrhius*. *Biotechnol. Bioeng*. 33: 912-914.
29. Lee, K. M. and S. Y. Lee (2001) Influence of ammonium phosphate on mycelial morphology during submerged cultivation of *Ganoderma lucidium*. *Kor. J. Mycol*. 29: 91-98.
30. Ebbole, D. J. (1998) Carbon catabolite repression of gene expression and conidiation in *Neurospora crassa*. *Fungal Genet. Biol*. 25: 15-21.
31. Choi, J. H., S. Y. Kim, D. K. Oh, and J. H. Kim (1998) Optimization of culture conditions for production of a high viscosity polysaccharide, methylans, by *Methylobacterium organophilum* from methanol. *Kor. J. Appl. Microbiol. Biotechnol*. 26: 244-249.
32. Lee, J. H. and I. Y. Lee (2001) Optimization of uracil addition for curdlan (β-1→3-glucan) production *by Agrobacterium* sp. *Biotech. Lett*. 23: 1131-1134.