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Effect of olive oil on the production of mycelial biomass and polysaccharides of *Grifola frondosa* under high oxygen concentration aeration

Chienyan Hsieh *, Chia-Jang Liu, Mei-Hua Tseng, Chaur-Tsuen Lo, Yuan-Chang Yang

Department of Biotechnology, National Formosa University, Yun-Lin 63208, Taiwan

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Abstract

In this research, the factors of oxygen concentration (21%, 30%, and 40%) and olive oil addition were studied on the production of mycelial biomass and polysaccharides of *Grifola frondosa* in a 5-L jar fermentor. The 40% oxygen was found to inhibit both cell growth and polysaccharide production with a higher rate of glucose consumption. The highest cell concentration was found at 9.29 g/L at day 9 with 1 vvm at 21% O_2. With 30% O_2 the inter-cellular polysaccharide (IPS) level increased earlier than 21% O_2 and reached the maximum concentration at day 8 with 21.9 mg/g. On the other hand, 21% O_2 reached the maximum concentration at day 11 with 19.5 mg/g. With 1% olive oil addition in 21% O_2 and 40% O_2, the production of mycelial biomass was enhanced and increased to 10.1 and 14.9 g/L, respectively, after 9 days' cultivation. And the extra-cellular polysaccharide (EPS) production increased from 0.7–0.9 g/L to 2.24 g/L and 3.00 g/L at day 13 with 21% O_2 and 40% O_2 aeration, respectively. In addition, the IPS increased rapidly and reached the maximum level of 28.2 mg/g at day 7 and this level remained till day 13 through the whole fermentation.

The scanning electron microscopy (SEM) of the mycelia and cell activity were studied to investigate the role of olive oil under high concentration of oxygen aeration. The vegetative cell was observed from both the 7-day-old culture and the 9-day-old culture from both 21% O_2 and 40% O_2 with olive oil addition. With 40% O_2 aeration, low growth activity and significant wrinkles appeared on the surface of mycelia on both 7-day-old culture and 9-day-old culture. The olive oil addition in media was observed to reduce the wrinkles appeared on the surface of mycelia under high concentration oxygen and made the cell be aging more slowly.

Keywords: *Grifola frondosa*; Oxygen concentration; Polysaccharides; Olive oil; SEM; Morphology

1. Introduction

Grifola frondosa is a Basidiomycete fungus belonging to the order *Aphyllopherales*, and family *Polyporaceae* [1]. *G. frondosa* (Maitake) is also called the king of mushrooms and the hen of the woods [1]. Maitake is used as a Chinese medicine called "keisho". "Shen nong ben cao jing" means that it has been frequently used for improving the ailment of spleen and stomach, calming the nerve and the mind, and treating the hemorrhoids. *G. frondosa* has become an increasingly popular food in recent years in Japan because of its bioactive ingredients. It contains a polysaccharide compound beta-glucan not found in other types of mushroom, and beta-glucan is reported to help strengthen the body's natural immune system and improve general health [2,3]. In addition to the health benefits, Maitake mushrooms have a distinctive rich, woody flavor and firm, meaty texture. Usually, the fruiting body of *G. frondosa* is dried to extend the shelf-life. Moreover, dry fruiting body of *G. frondosa* is also used in the production of health foods, including *G. frondosa* tea, whole *G. frondosa* powder, powders of hot water extracts of *G. frondosa*, *G. frondosa* granules, and *G. frondosa* drinks [4]. In a previous study, Suzuki et al. described the purification, general properties and substrate specificity against oxidized insulin B-chain of pepstatin-insensitive grifolisin from *G. frondosa*. They also described the isolation and structural analysis of the cDNA (*gfrF*) coding for grifolisin and compared the deduced amino acid sequences with those of other sedolisins [5].

The fruiting body and liquid-cultured mycelium of this mushroom have been reported to contain effective anti-tumor polysaccharides from various fractions. These polysaccharides have been identified as glucans (e.g. β-1,6- and β-1,3-) [6–8]. The

* Corresponding author.

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D-fraction from the dried powder of fruiting body of *G. frondosa* was observed to induce angiogenesis in vivo and to enhance the proliferation and migration of human vascular endothelial cell in vitro. In addition, the D-fraction also increased plasma vascular endothelial growth factor concentration significantly [9]. Moreover, the D-fraction, a β-glucan extracted from the fruiting bodies of *frondosa*, activated cellular immunity expressing the effects of anti-tumor [6–8]. Besides, the anti-tumor capability of D-fraction was related to its control the balance between T lymphocyte subsets Th-1 and Th-2. Furthermore, the D-fraction decreased the activation of B cells and potentiated the activation of helper T cells, resulting in enhancing cellular immunity [10]. In this study, five groups of polysaccharides were prepared from mycelium extract by submerged culture of *G. frondosa* except D-fraction from the fruiting body. Polysaccharides with 500 and 770 kDa had antioxidant and free radical scavenging activities after UV irradiation. The molecular weight at 1650 kDa had shown to increase the proliferation of fibroblasts approximately [11].

According to the previous study, different kinds of plant oil had been selected to accelerate mycelial growth of some mushroom species and proved to have a stimulatory effect [12]. In addition, many researchers had also reported that fatty acid, oil and surfactant promoted the production of fungal metabolites [13,14]. Moreover, based on the previous study, at higher initial oxygen transfer level, a maximal cell concentration (15.62 g/L) had been obtained, as well as a maximal intracellular polysaccharide (IPS) production in the submerged fermentation of *Ganoderma lucidum* [15]. On the other hand, some studies reported that a lower dissolved oxygen supply would enhance the polysaccharide production, and the production of extra-cellular polysaccharides (EPS) and the IPS content at 10% dissolved oxygen tension (DOT) were higher than those at 25% DOT [15]. The more compact pellet was favorable for *exo*-biopolymer production. Under extremely low and high aeration conditions, severe deformations of pellets were observed at the later stages of fermentation associated with a decrease in morphological parameters [16].

So far, the role and effects of fatty acid on cell growth and polysaccharide production by *G. frondosa* have not been reported. In this research, we demonstrated the effect of olive oil addition under high concentration oxygen aeration in a 5-L jar fermentor on the mycelial morphology, mycelial activity, cell growth and polysaccharides production by *G. frondosa*.

2. Materials and methods

2.1. Organism

In this study, *G. frondosa* was obtained from Taiwan Agricultural Research Institute, Wufeng, Taiwan. Then, the cultures were maintained on potato–agar–dextrose slopes that were inoculated and incubated at 25 °C for 14 days, and stored at 4 °C. The mycelium was activated by culturing on a modified agar plate (4 g/L yeast extract, 10 g/L malt extract, 2 g/L glucose, 1.0 g/L molasses, 10 mL/L mineral salt solution, and 15 g/L agar) at 25 °C for 7 days. And the experimental inoculums were prepared in 250 mL Erlenmeyer flasks containing 100 mL of the medium with four units of a cutter square of activated mycelia. Mycelia agar squares (5 mm × 5 mm) were obtained by a self-designed cutter to use as the inoculums in a shake-flask culture. The media consisted of the following components: 4 g/L YE, 10 g/L ME, 2 g/L glucose, 1 g/L molasses, and 10 mL mineral salt solution. And the mineral salt solution contains 120 g/L $MgSO_4 \cdot 7H_2O$, 6 g/L NaCl, 20 g/L KH_2PO_4, 20 g/L $CaCl_2$, 10 g/L $FeSO_4 \cdot 7H_2O$, and 1.8 g/L $ZnCl_2$. The flasks were incubated on a New Brunswick rotary shaker (Model G24) at 25 °C, 150 rpm for 7 days. Then, the mycelium was homogenized by a sterilized blender for 30 s to be the inoculums in the following experiments.

2.2. Jar fermentor

The fermentations were implemented in a 5-L jar fermentor (Biotop, BTF-A-5*L*, Taiwan) with a working volume of 3 L and 10% inoculum at 25 °C for 5 days. In this study, the pH of medium was adjusted to five prior to sterilization; the agitation rate remained at 250 rpm, and the culture was aerated at a rate of 1 vvm. Besides, the 40% oxygen was derived from two sources of gas, one in which the air was calculated as 21% oxygen and the other in which the 95% oxygen was produced by an oxygen generator (EYELA, Model SO-004, Japan). And three oxygen supplies were: (1) 1 vvm 21% oxygen, (2) 1 vvm 30% oxygen, and (3) 1 vvm 40% oxygen.

2.3. Analytical methods

2.3.1. Cell concentration

The cell concentration was termed as the dry weight per unit volume. A fermentation broth in the amount of 10 mL was obtained and subjected to centrifuge at 4185 × *g* (6000 rpm) for 15 min (HERMLE, model Z200A). Then, the sediment produced at 4185 × *g* was washed, resuspended, and centrifuged twice with 10 mL distilled water. After that, the sediment was frozen by drying to a constant weight.

2.3.2. Concentration of glucose

The residual glucose in the fermentation broth was determined by an HPLC with an ionic exchange column. And the mycelia cell was precipitated by centrifugation (HERMLE, model Z160M) at 10,290 × *g* (10,000 rpm) for 10 min, after which the supernatant was collected. Then, the supernatant was filtrated through a 0.45 μm cellulose acetate filter, after which an internal standard (lactose) was added. And, a 20 μL of filtrate sample was injected into the HPLC by using an ionic exchange column (CARBO Sep COREGEL 87H3, TRANSGENOMIC), which was placed in a column oven (COLBOX) at 55 °C. The 0.008 N H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL/min, and the effluent was monitored by an RI detector (RI-1530, Jasco, Japan).

2.3.3. Determination of polysaccharides

In order to determine the extra-cellular polysaccharides, the fermentation broth filtrate was added to four volumes of 95% ethanol and left overnight at 4 °C to precipitate the crude polysaccharides. Then, the precipitated polysaccharides were collected by centrifugation (HERMLE, model Z160M) at 10,000 rpm for 10 min, after which they were dried to remove the residual ethanol with a freezing dryer. According to the method developed by Dubois et al., the total polysaccharide content was determined by a phenol–sulfuric acid assay [17]. And the filtered mycelia were washed twice with the same volume of distilled water, and collected for the determination of intracellular polysaccharides and cell concentration. The procedure for IPS concentration in this study was the same as that mentioned above except that prior to ethanol precipitation and 100 mg of the freeze–dried mycelia was ground into powder and extracted with 10 mL of 121 °C distilled water for 30 min [18].

2.4. Scanning electron microscopy

The freeze–dried mycelia of *G. frondosa* was coated with gold in an atmosphere of argon in an ion sputtering device (JFC-1100E, JEOL, Japan), at a current of 10 mA for 2 min. Coated material was examined and photographed with a scanning electron microscopy (JOEL 5400, Japan) at 20 kV.

3. Results and discussion

3.1. Effect of oxygen concentration

Three different aeration treatments were used for the cell growth and polysaccharide production by *G. frondosa* in a 5-L jar fermentor. With 1 vvm of 21% O_2, a rapidly cell growth was observed to reach the highest cell concentration of 9.30 g/L at day 9 (Fig. 1(a)), after which the cell concentration decreased to 6.41 g/L at the end of the fermentation period (13 days). It was worth to mention that a diauxic growth was found with 21% O_2 and 30% O_2 cultivation. Higher oxygen concentrations (30% or 40%) were supplied at 1 vvm, and the cell concentrations were found to be lower than that with 1 vvm of 21% O_2. And the cell concentration reached 4.28 and 3.81 g/L for the aeration of 30% O_2 and 40% O_2, respectively. On the other hand, high oxygen concentration might exhibit a toxic effect on the cell and inhibit the cell growth. These phenomena were similar to the oxidative stress occurring in the cell. According some studies, oxidative stress was considered to play a prominent role in the causation of many diseases such as inflammation, aging and cancer [2,4,7].

The correlation between glucose consumption and cell growth was inverse under various oxygen concentrations (Fig. 1(b)). The glucose consumption was rapid under 40% O_2 aeration. The glucose concentration decreased to 1.46 g/L after 7 days (Fig. 1(b)). Moreover, the 21% O_2 and 30% O_2 were depleting glucose at day 9 and at day 13, respectively. Higher glucose consumption might be due to higher oxygen concentration for the extra energy required to metabolize cell growth and avoid oxygen toxicity. The profiles of the correlation between pH with glucose consumption showed that the lower the pH was, the more the glucose consumption was. The highest rate of pH decrease was found with 40% O_2 and the pH was decreased to 2.6 at day 6. At the mean time, the highest rate of glucose consumption was also found with 40% O_2. A lower rate of pH decrease was found with 21% O_2 while the lower rate of glucose consumption was observed. It was believed that metabolite the carbon source (glucose) as the energy source will produce organic acid and result in lower pH in the fermentation broth. On the other hand, the depletion of carbon source will shift to use nitrogen source as energy source, and then produce ammonium material and result in higher pH of fermentation broth.

The extra-cellular polysaccharides (EPS) were not shown any significant difference under three different oxygen concentrations and mild oscillation of EPS was observed in the range of 0.7–0.9 g/L (Fig. 1(c)). The higher initial EPS might be due to the presence of polysaccharides in the media and the EPS was part of nutrient for the cell growth. Analyzing the nitrogen source from corn steep powder, the polysaccharides in the media were confirmed. With 30% and 40% O_2 the oscillation of EPS was found and it might be due to cell lyses and release of cell contents, including polysaccharides to the broth. At the mean time, the carbon source began to be close to depletion (Fig. 1(b)), and the decreasing polysaccharides might have been hydrolyzed and utilized by the cell again. We found that both the consumption of polysaccharide from the nutritious source and the production of the polysaccharide from *G. frondosa* occurred simultaneously. It was noteworthy that the combination of dissolved oxygen concentration with available carbon source played an important role in higher EPS production. These results were also in good agreement with the previous study [14]. On the other hand, the inhibitory effect of high dissolved oxygen concentration was observed on polysaccharide production in *Aureobasidium pullulans* [19].

In this study, the specific inter-cellular polysaccharide (IPS) was used as IPS production and it was expressed in terms of mg

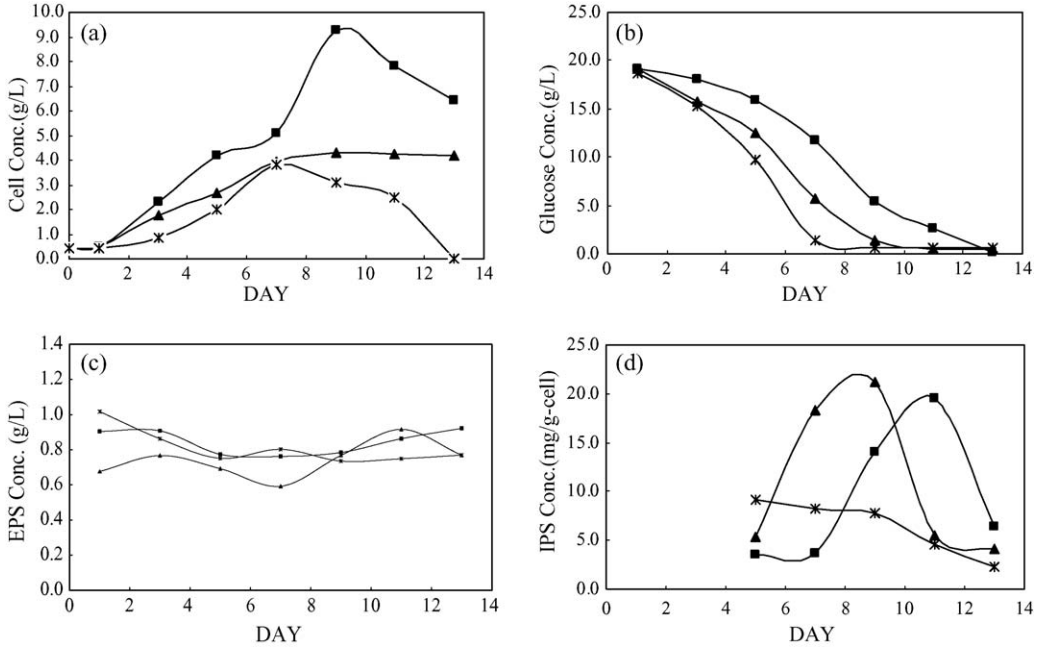

Fig. 1. Effect of oxygen concentration on the profiles of (a) cell growth, (b) glucose utilization, (c) extra-cellular polysaccharide production, and (d) inter-cellular polysaccharide production by *G. frondosa* in 5-L jar fermentor. (■) 21% oxygen, (▲) 30% oxygen, and (✳) 40% oxygen.

per unit of cell weight (mg/g). And the IPS began to increase after 5 days' and 7 days' cell cultivation with 30% O_2 and 21% O_2, respectively (Fig. 1(d)). Then the IPS decreased depending on the level of available glucose (Fig. 1(b) and (d)). With 30% O_2 the IPS level increased earlier than 21% O_2 and reached the maximum concentration at day 8 with 21.9 mg/g. On the other hand, 21% O_2 reached the maximum concentration at day 11 with 19.5 mg/g (Fig. 1(d)). The early increase IPS might be due to the inductive effect by oxygen. However, this inductive effect was overcome by oxidation and toxicity of a high oxygen concentration. With 40% O_2 the level of IPS was very low and slow decrease with the period of cultivation. The polysaccharide had been demonstrated to be free of free-radical-induced neuronal cell toxicity [4]. The low level of IPS in the fermentation was because 40% O_2 aeration might inhibit polysaccharide production or because the consumption of IPS was to protect cell constituents from the toxicity of a high oxygen concentration.

3.2. Effect of olive oil addition

Based on the results of the previous study, the olive oil addition had the best stimulatory effect on the polysaccharide production by *G. frondosa*. Then 1% olive oil was added to media under aerations of 21% O_2 and 40% O_2 to grow *G. frondosa* in 5-L jar fermentor. Both 21% O_2 and 40% O_2 aeration with oil addition formed pellet after 3 days' cultivation and a higher cell concentration was found with less foam of fermentation broth (Fig. 2(a)). It was worth to mention that the mycelium was able to grow very well under 40% O_2 with oil addition. The highest cell concentration was found at 14.9 and 10.1 g/L at day 9 with 40% O_2 and 21% O_2, respectively (Fig. 2(a)). Comparing with the sample without oil addition, a slightly increased cell concentration was found in olive oil addition under 21% O_2 aeration. However, four times of cell concentration was found in olive oil addition under 40% O_2 aeration. That the cell growth of *G. frondosa* increased significantly might be due to the effect of oil protection from oxygen toxicity and the high dissolved oxygen available from 40% O_2 aeration for cell growth. The rate of glucose consumption was lower than that without oil addition (Fig. 2(b)). The depletion of glucose was found at day 13 both with 21% O_2 and 40% O_2. However, the profiles of pH from the culture media with oil addition were different from those in the previous results without oil addition. On the early 8 days, the pH with 40% O_2 decreased more rapidly than that with 21% O_2 and then the pH remained at around 3 both with 21% O_2 and 40% O_2. The low pH of broth might be that the glucose was not depleted yet at the later stage of fermentation. On the other hand, olive oil could be served as the second carbon source instead of using nitrogen source for energy metabolite.

In the media with 1% olive oil addition under for both 21% O_2 and 40% O_2 aeration, the EPS production significantly increased. The EPS significantly increased after 7 days' cultivation. The EPS concentration increased from 0.7–0.9 g/L to 2.24 g/L and 3.00 g/L at day 13 with 21% O_2 and 40% O_2 aeration, respectively (Fig. 2(c)). After 11 days' cultivation, the high EPS production broth was sampled out from the fermentor and placed under room temperature statically. After about 1 h, the jelly-formed broth was observed both in 21% O_2 and 40% O_2 with oil addition. The result of the enhancement of EPS production with oil addition was also in good agreement with the previous study [20,21]. According to some studies, the low pH of broth resulted from oil addition might favor the EPS to produce continuously [22]. With 1% oil addition under 21% O_2 aeration, the IPS significantly increased (Fig. 2(d)). After 5 days' cell cultivation, the IPS began to increase with oil addition at 21% O_2 aeration. Then the IPS increased rapidly and reached the maximum level of 28.2 mg/g at day 7 and this level remained till day 13 through the whole fermentation (Fig. 2(d)). However,

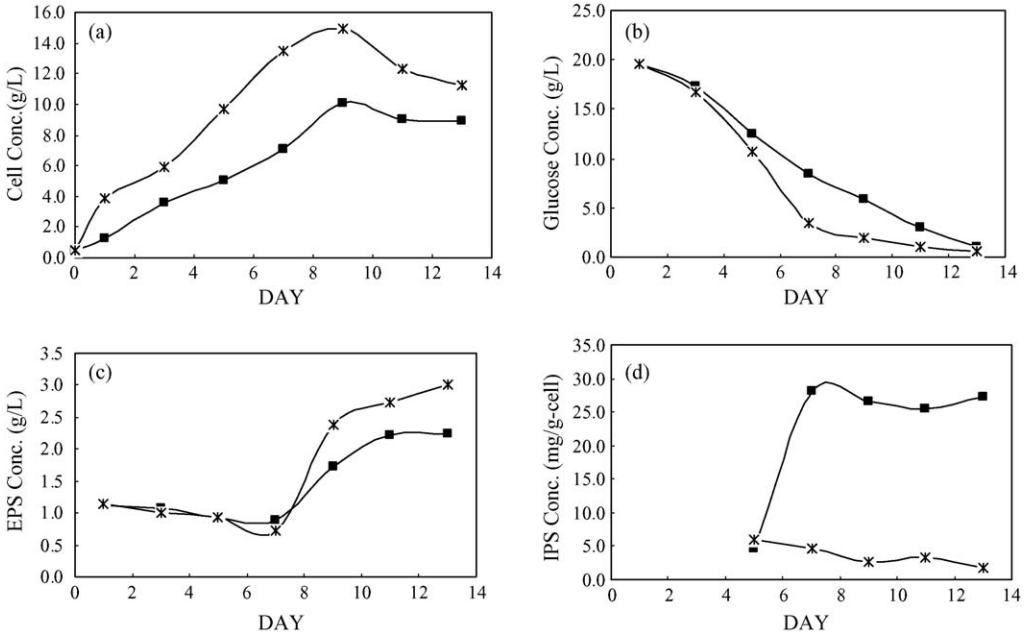

Fig. 2. (a) The cell growth, (b) glucose utilization, (c) extra-cellular polysaccharide production, and (d) inter-cellular polysaccharide production of *G. frondosa* in 5-L jar fermentor under 21% O_2 and 40% O_2 with 1% olive oil addition. (■) 21% oxygen w/oil and (-✳-) 40% oxygen w/oil.

the IPS was low with oil addition at 40% O_2 aeration, and the IPS production was below 10 mg/g. We found that 1% olive oil addition only enhanced the IPS production under 21% O_2 but not 40% O_2, and the mechanism to enhance IPS production under 21% O_2 was still unclear. A possible reason is that high oxygen concentration might inhibit the formation of cell wall and result in leaking polysaccharides to broth. To investigate the role of olive oil to play under high concentration of oxygen aeration, then the SEM and cell activity were studied.

3.3. Morphology and cell activity of mycelia

The morphology of the 7-day-old and 9-day-old mycelia from 5-L fermentor cultivation under different oxygen concentration aeration was observed by scanning electron micrographs with magnification of 1000 times (Fig. 3). With 21% O_2 aeration, the mycelia of pellet were thick and intertwined with each other after 7 days' cultivation (Fig. 3(a)). Then broken filamentous mycelia were found to be closely clustered together at outlet of pellet after 9 days' cultivation. At the mean time, some irregular shapes of the mycelia were observed and that seemed to lyses (Fig. 3(d)). A similar result was reported on the observation of pellet morphology of *Cordyceps militaris* [16]. With 40% O_2 aeration, the mycelial morphology was significantly different from that with 21% O_2 aeration. With 40% O_2 aeration, a greater proportion of the wrinkly mycelia surface was observed and the polysaccharides extra-cellular material was found over the mycelia surface forming a thick layer adhesion (Fig. 3(b) and (e)). In addition, the pellets found in 40% O_2 had very smooth surface and can be observed by eye. This extra-cellular material might be due to the mycelia lyses from which the polysaccharide leakage adhered to the surface of other mycelia. With olive oil addition, a cluster of sticky-like material was found to cover the surface of mycelia (Fig. 3(c)). This sticky-like material was also found to reduce the mycelia lyses at the later stage of fermentation (Fig. 3(f)). This sticky-like material might be the fatty acid from olive oil or the mixture of olive oil and metabolite from *G. frondosa*. However, this protection effect was in agreement on the previous results.

The activities of fungal mycelia under different oxygen aeration with or without oil addition were monitored by weight per volume as well as the inter-cellular polysaccharide of cell in a shaking flask with non-oil media. The inocula were prepared from the 7-day-old and the 9-day-old culture under 21% O_2, 40% O_2, and 40% O_2 with 1% olive oil addition in a 5-L fermentor. The EPS was not taken into count at this stage because the residual polysaccharide from nitrogen source would influence the production under poor cell growth. After 7 days' cultivation, the cell growth from the inocula of 21% O_2 did not show any significant difference in cell concentration between the 7-day-old inocula and the 9-day-old inocula and the cell concentration was 1.5 ± 0.25 g/L (Fig. 4). The IPS production from 7-day-old inocula was quite different from that from the 9-day-old inocula. The amounts of IPS in 9-day-old inocula were significantly higher than those in 7-day-old inocula (Fig. 5). With the inocula from 40% O_2, both 7-day-old inocula and 9-day-old inocula were resulted in very low growth and the IPS was not able to be measured with such few cell production. On the other hand, the inocula from the media with olive oil addition under 40% O_2 aeration, the cell growth markedly increased, therefore, the IPS production became higher than those from the media without oil addition. Additionally, the 9-day-old inocula were superior to the 7-day-old inocula in both cell growth and IPS production (Figs. 4 and 5).

In conclusion, the mycelial growth of *G. frondosa* was inhibited and resulted in low polysaccharides production under 40% O_2 aeration in 5-L fermentor cultivation. However, 1% olive oil addition would enhance the cell growth and polysaccharides production. The morphology at different phases of mycelia was monitored by scanning electron micrographs. A smooth mycelia

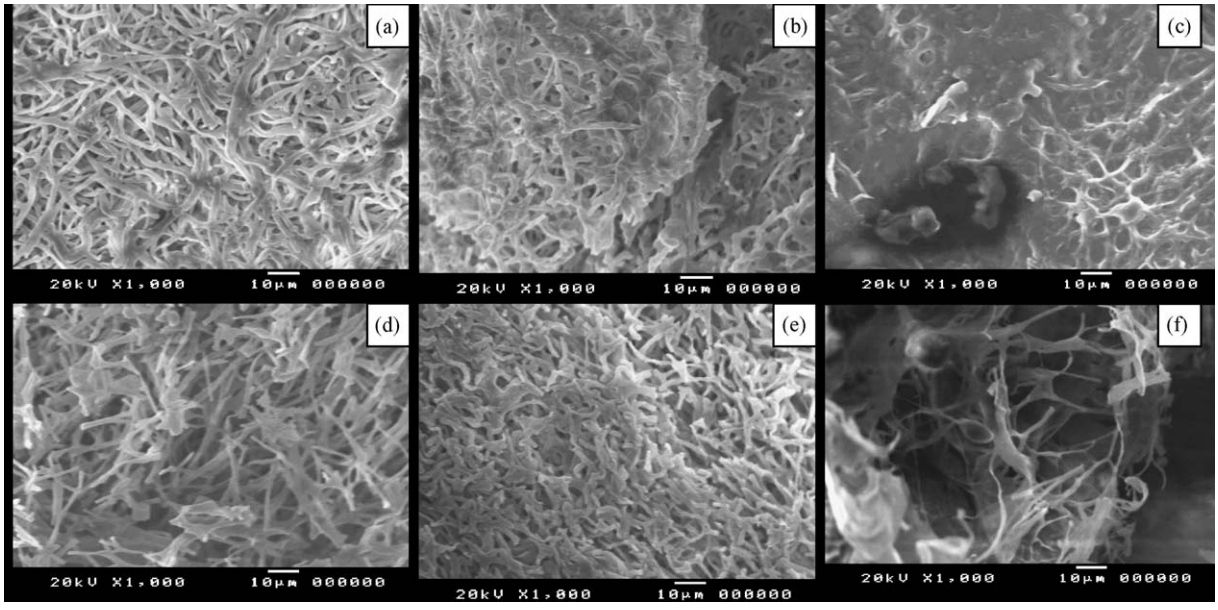

Fig. 3. Scanning electron micrographs (X1000) of mycelial growth (a) 7-day-old culture under 21% O_2; (b) 7 day-old culture under 40% O_2; (c) 7-day-old culture under 40% O_2 with oil addition; (d) 9-day-old culture under 21% O_2; (e) 9-day-old culture under 40% O_2; (f) 9-day-old culture under 40% O_2 with oil addition.

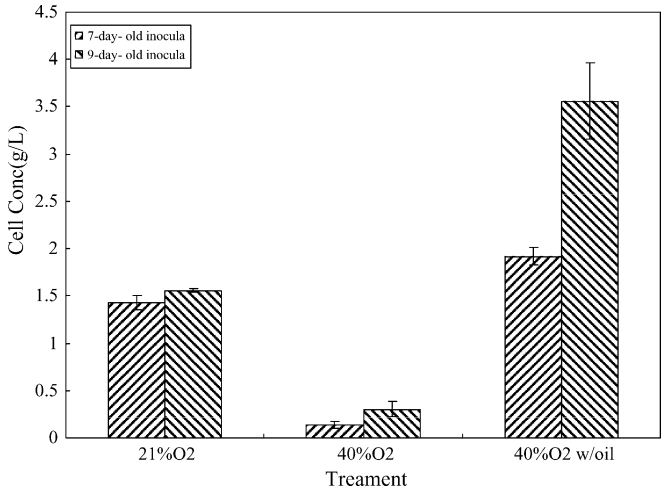

Fig. 4. Cell growth in shaking flask using 7-day-old-inocula and 9-day-old-inocula from 5-L fermentor cultivation under different oxygen concentration aeration.

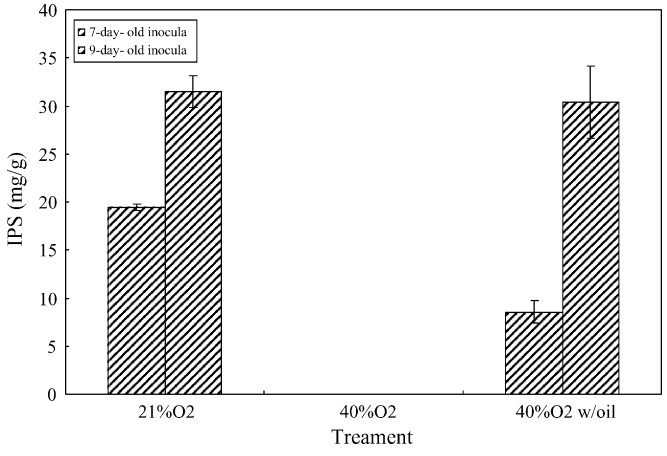

Fig. 5. IPS production in shaking flask using 7-day-old inocula and 9-day-old inocula from 5-L fermentor cultivation under different oxygen concentration aeration.

surface was found at 7-day-old culture and discontinuous and surface wrinkle mycelia was found at 9-day-old culture in 5-L fermentor with 21% O_2 aeration. The vegetative cell was received from both the 7-day-old culture and the 9-day-old culture. With 40% O_2 aeration, significant wrinkles appeared on the surface of mycelia on both 7-day-old culture and 9-day-old culture. In other words, the stationary-phase cell would be aging rapidly under this circumstance; therefore, it was not suitable to support continuous growth for the usage of inocula. On the whole, the olive oil addition in media was observed to reduce the wrinkles appeared on the surface of mycelia under high concentration oxygen and made the cell be aging more slowly.

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References

[1] Stamets P. Growing gourmet and medicinal mushrooms. Berkeley, CA: Ten Speed Press; 1993.
[2] Adachi K, Nanba H, Kuroda H. Potentiation of host-mediated antitumor activity in mice by beta-GLUCAN obtained from *Grifola frondosa* (Maitake). Chem Pharm Bull 1987;35:262–70.
[3] Nanba H. Antitumor activity of orally administered D-fraction from Maitake mushroom (*Grifola frondosa*). J Naturopath Med 1993;1:10–5.
[4] Mizuno T, Sakai T, Chihara G. Health foods and medicinal usages of mushrooms. Food Rev Int 1995;11:69–81.
[5] Suzuki N, Nishibori K, Oodaira Y, Kitamura SI, Michigami K, Nagata K, et al. A member of the sedolisin family produced by the fungus *Grifola frondosa*. Phytochemistry 2005;66:983–90.
[6] Mizuno T, Ohsawa K, Hagiwara N, Kuboyama R. Fractionation and characterization of antitumor polysaccharides from Maitake *Grifola frondosa*. Agric Biol Chem 1986;50:1679–88.
[7] Ohno N, Adachi Y, Suzuki I, Sato K, Oikawa S, Yadomae T. Characterization of the antitumor glucan obtained from liquid-cultured *Grifola frondosa*. Chem Pharm Bull 1986;34:1709–15.
[8] Shigesue K, Kodama N, Nanba H. Effects of Maitake (*Grifola frondosa*) polysaccharide on collagen-induced arthritis in mice. Jpn J Pharmacol 2000;84:293–300.
[9] Matsui K, Kodama N, Nanba H. Effects of Maitake (*Grifola frondosa*) D-fraction on the carcinoma angiogenesis. Can Lett 2001;172:193–8.
[10] Inoue A, Kodama N, Nanba H. Effect of Maitake (*Grifola frondosa*) D-fraction on the control of the T lymph node Th-1/Th-2 proportion. Biol Pharm Bull 2002;25:536–40.
[11] Lee BC, Bae JT, Pyo HB, Choe TB, Kim SW, Hwang HJ, et al. Biological activities of the polysaccharides produced from submerged culture of the edible Basidiomycete *Grifola frondosa*. Enzyme Microb Technol 2003;32:574–81.
[12] Schisler LC, Volkoff O. The effect of safflower oil on mycelial growth of *Boletaceae* in submerged liquid cultures. Mycologia 1977;69:118–25.
[13] Fukushima Y, Itoh H, Fukase T, Motai H. Stimulation of protease production by *Aspergillus oryzae* with oils in continuous culture. Appl Microb Biotechnol 1991;34:586–90.
[14] Kojima I, Yoshikawa H, Okazaki M, Terui Z. Studies on riboflavin production by *Eremothecium ashbyii*. J Ferment Technol 1972;50:716–23.
[15] Tang YJ, Zhong JJ. Role of oxygen supply in submerged fermentation of *Ganoderma lucidum* for production of Ganoderma polysaccharide and ganoderic acid. Enzyme Microb Technol 2003;32:478–84.
[16] Park JP, Kim YM, Kim SW, Hwang HJ, Cho YJ, Lee YS, et al. Effect of aeration rate on the mycelial morphology and *exo*-biopolymer production in *Cordyceps militaris*. Process Biochem 2002;37:1257–62.
[17] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350–6.
[18] Hsieh C, Hsu TH, Yang FC. Production of polysaccharides of *Ganoderma lucidum* (CCRC36021) by reusing thin stillage. Process Biochem 2005;40:909–16.
[19] Lazaridou A, Roukas T, Biliaderis CG, Vaikousi H. Characterization of pullulan produced from beet molasses by *Aureobasidium pullulans* in a stirred tank reactor under varying agitation. Enzyme Microb Technol 2002;31:122–32.
[20] Yang FC, Ke YF, Kuo SS. Effect of fatty acids on the mycelial growth and polysaccharide formation by *Ganoderma lucidum* in shake flask cultures. Enzyme Microb Technol 2000;27:295–301.
[21] Park JP, Kim SW, Hwang HJ, Cho YJ, Yun JW. Stimulatory effect of plant oils and fatty acids on the *exo*-biopolymer production in *Cordyceps militaris*. Enzyme Microb Technol 2002;31:250–5.
[22] Hsieh C, Tsai MJ, Hsu TH, Chang DM, Lo CT. Medium optimization for polysaccharide production of *Cordyceps sinensis*. Appl Biochem Biotech 2005;120:145–57.