

Effects of Nitrogen from Different Sources on Mycelial Biomass and Polysaccharide Production and Pellet Morphology in Submerged Cultures of *Grifola frondosa*

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The effects of nitrogen in the medium on the production of mycelial biomass, extracellular polysaccharides (EPS), and intracellular polysaccharides (IPS) was investigated in submerged cultures of *Grifola frondosa*. In addition, the effects on pellet morphology were examined. The maximum production levels of mycelial biomass (2.32 g/L), EPS (1.58 g/L), and IPS (29.1 mg/L) were obtained when the nitrogen sources in the medium were yeast extract, malt extract, and peptone, respectively. Using yeast extract as the nitrogen source yielded the maximum mycelial biomass, and morphological characterization revealed a composition of 47% large pellets (fraction L), 20% small pellets (fraction S), and 33% adhesive mycelia (fraction A). The maximum circularity value and the minimum roughness value of the pellets were observed using yeast extract cultures. Both the compactness (0.53) and circularity (0.15) of the pellets were the lowest among the seven types of nitrogen sources, but the roughness (2.86) was the highest in malt extract, which was the nitrogen source that resulted in maximum polysaccharide production. The results revealed that the production levels of mycelial biomass, EPS, and IPS of *G. frondosa* were associated with changes in pellet morphology due to the source of nitrogen in the medium.

Keywords: *Grifola frondosa*; Nitrogen sources; Polysaccharide; Morphological; Pellets

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INTRODUCTION

Grifola frondosa is a *Basidiomycete* fungus belonging to the order *Aphylopherales* and the family *Polyporaceae*. *Grifola frondosa* is a popular culinary and medicinal mushroom due to its good flavor, medicinally potent variety, and exceptional texture. The bioactivities of *G. frondosa* fruiting body and submerged-cultured mycelia are of great interest to market them as functional foods (Wu *et al.* 2016). Physiologically active polysaccharides can also be obtained from commercially cultivated mushrooms that usually require three months to culture due to their fruiting body, whereas the submerged culture of *G. frondosa* can be employed to grow mycelia with a shorter growth time and better product quality (Shih *et al.* 2008; Wu *et al.* 2021). Therefore, submerged fermentation of *G. frondosa* is a promising alternative for the efficient production of mycelial biomass and polysaccharides. Most of the active polysaccharides from *G. frondosa* can be isolated *via* ethanol precipitation and hot water extraction of its submerged fermentation broth and mycelium (Mizuno and Zhuang 1995; Lin 2011). Among these polysaccharides, β -(1-3)-D-glucan exhibits antitumor effects (Kodama *et al.* 2005; Masuda *et al.* 2009a), cancer cell apoptosis (Cui *et al.* 2007; Shi *et al.* 2007; Mao *et al.* 2015), liver

function protection (Kubo and Nanba 1997; Wang *et al.* 2010), myelosuppression reduction (Masuda *et al.* 2009b), anti-atherosclerotic effects (Mori *et al.* 2008; Ding *et al.* 2016), control of diabetes (Preuss *et al.* 2007; Lo *et al.* 2008; Li *et al.* 2019), induction of vascular endothelial growth factor (Lee *et al.* 2008), and anti-HIV activity (Nanba *et al.* 2000; Gu *et al.* 2007). The optimal conditions for the production of polysaccharide *via* submerged cultures of *G. frondosa* have recently received increased attention (Huang and Liu 2007; Shih *et al.* 2008; Tao *et al.* 2018)). Past reports have mainly focused on extracellular polysaccharides (EPS) rather than intracellular polysaccharides (IPS). However, the optimal culture factors for the production of both polysaccharide types should be considered simultaneously.

Fungal morphology is an important determinant of the rheological properties of the fermentation broth, and controlling morphology is highly desired (Park *et al.* 2002a; Cui *et al.* 2016). Fungal morphology is influenced by many factors, including medium composition (Paul *et al.* 1999; Ahamed and Vermette 2009), dissolved oxygen concentration (Park *et al.* 2002b; Veiter *et al.* 2018), agitation speed (Chen *et al.* 2008; Cui *et al.* 2016; Veiter *et al.* 2018), and pH (Kim *et al.* 2003; Ahumada-Rudolph *et al.* 2019). In addition, many studies have reported the relationship between specific fungal morphology and metabolites (Wagner *et al.* 2004; Anteck *et al.* 2016). However, little is understood about the growth of *G. frondosa* in submerged cultures. In particular, the relationship between morphology, nitrogen sources, and polysaccharides remains poorly understood. This study is the first report that has examined the pellet morphology of the submerged culture of *G. frondosa*, its biomass, and polysaccharide production.

This study aimed to investigate the effect of various nitrogen sources on the production of mycelial biomass and polysaccharides by *G. frondosa* and characterized the pellets and mycelia morphology to determine the most favorable form for polysaccharide production.

EXPERIMENTAL

Strain, Culture, and Inoculation

Grifola frondosa was obtained from the Culture Collection and Research Centre of the Food Industry Research and Development Institute (Hsinchu, Taiwan; CCRC 36911), maintained on potato dextrose agar (PDA) slants, and subcultured every month. The slants were incubated at 25 °C for 21 d and then stored in a refrigerator at 4 °C for further use. The seed culture transferred from the slant was grown on PDA in a Petri dish at 25 °C for 21 d, and 5 discs of PDA (5-mm diameter agar) containing *G. frondosa* were punched out and inoculated into 100 mL of potato dextrose broth (PDB) medium in a shaker at 100 rpm at 25 °C for 14 d. Subsequently, the flask culture experiments were performed in 500-mL flasks with 10 wt% of the seed culture at an initial pH of 5.5 at 25 °C, and the samples were cultured for 30 d (for static cultures) or 14 d (for shaking cultures) at 100 rpm. The original fermentation medium consisted of the following ingredients (g/L): glucose, 30; yeast extract, 8; MgSO₄·7H₂O, 0.5; MnSO₄, 0.2; K₂HPO₄, 0.5.

Nitrogen Sources

To identify the most suitable nitrogen sources for the production of biomass, 0.8% of the nitrogen source in the original fermentation medium were converted to 0.8%

inorganic nitrogen (ammonium sulfate [(NH₄)₂SO₄], ammonium oxalate [(NH₄)₂C₂O₄], ammonium chloride [NH₄Cl]), 0.8% organic nitrogen (malt extract, yeast extract, and peptone), and a mixture of nitrogen sources (0.2% peptone added to 0.6% yeast extract) in shaking cultures.

Analytical Methods

Biomass fractionation of the components and pH values

The biomass was separated into fractions L (retained by a sieve with 2.0 mm pores, > 10-mesh), S (passed through both sieves, < 10-mesh), and A (adhered to the internal wall of the flask) (Wagner *et al.* 2004). Each fraction was centrifuged (Hitachi CR22E, Tokyo, Japan) at 6000 rpm for 20 min. The obtained pellets were regarded as mycelia and washed twice with three-fold volumes of distilled water. Subsequently, the sediment was freeze-dried to a constant weight. In addition, the obtained supernatants were filtered through a pre-weighed Whatman filter paper (No. 5, Whatman International Ltd., Maidstone, England). Next, the pH values were measured, and the exopolysaccharides were precipitated from the filtrates.

Production of EPS and IPS polysaccharides

The filtrates were precipitated using four-fold volumes of 95% ethanol at 4 °C for 12 h, centrifuged at 8000 rpm for 30 min, and lyophilized to obtain crude EPS. To obtain IPS, 1 g of lyophilized mycelium was mixed with 10 mL of distilled water, heated at 100 °C for 3 h, and centrifuged at 6000 rpm for 15 min. Each biomass fraction was extracted. The EPS and IPS quantities were determined using the phenol-sulfuric acid assay according to Dubois *et al.* (1956).

Estimation of total nitrogen

Total nitrogen was estimated using the micro Kjeldahl method (Allen *et al.* 1974). For each sample, three replicates were analyzed, and the mean values were obtained.

Morphological characterization

The morphological properties of the samples collected were evaluated using an image analyzer (Simple PCI) with software (Hamamatsu Corp., version 5.1, Bridgewater, NJ, USA) linked to a light microscope (Olympus, Center Valley, PA, USA) equipped with an MIC-D camera. For each sample, the morphology of 20 pellets was characterized by measuring the area and perimeter of the pellet core and the maximum diameter of the pellet. Then, their mean diameter, circularity, roughness, and compactness were characterized based on pellet morphology. A magnification of 20× was used. The circularity or shape factor was estimated as the ratio of the Fieret's minimum to maximum diameters of the pellets or aggregates. The compactness was estimated as the ratio of the projected areas of the hyphae in a clump to the projected convex area of that clump; the projected convex area being the area after filling the internal voids and concavities in the clump's external perimeter. In addition, the roughness (*R*) was measured using Eq. 1 (Riley *et al.* 2000; Park *et al.* 2002a,b):

$$R = (\text{pellet / aggregate perimeter})^2 / (4 \times \text{pellet area}). \quad (1)$$

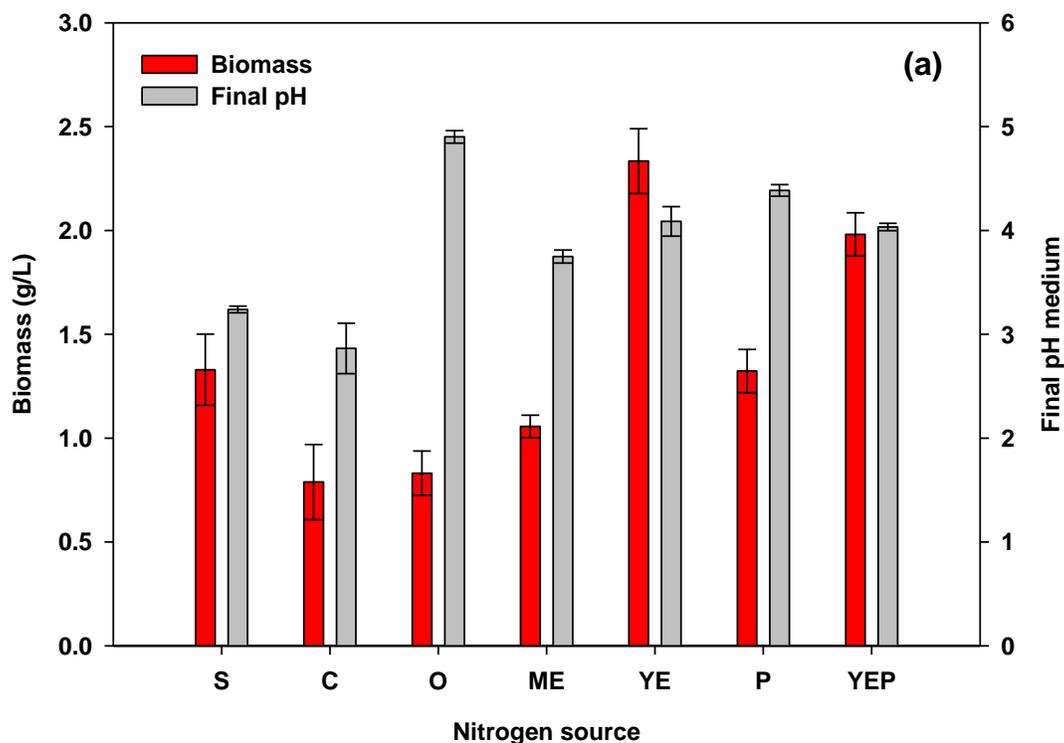
Image analyses

First, the freeze-dried pellet was placed in a dish of the stereo microscope (LABOMED CZM6, Labomed, Los Angeles, CA, USA). Next, the freeze-dried mycelia were coated with gold in an atmosphere of argon at a current of 10 mA for 2 min using an ion-sputtering device (JFC-1100E, JEOL Ltd., Tokyo, Japan). The coated materials were examined, and images were acquired using scanning electron microscopy (JOEL 5400; JEOL Ltd., Tokyo, Japan) at 20 KV (Hsieh *et al.* 2006).

RESULTS AND DISCUSSION

Effect of Nitrogen

Grifola frondosa was cultured using three types of inorganic (ammonium sulfate, ammonium chloride, and ammonium oxalate), organic (malt extracts, yeast extracts, and peptone), and dual (0.6% yeast extracts plus 0.2% peptone) nitrogen sources. The mycelia, EPS and IPS production, and the final pH medium are presented in Fig. 1. Among the sources examined, yeast extracts yielded the highest mycelial biomass (2.32 g/L), which was noticeably higher than the nitrogen content (1.30 g/L) produced from ammonium sulfate in the medium. A similar result was previously reported for nitrogen utilization by *G. frondosa* (Lee *et al.* 2004; Shih *et al.* 2008). In much previous research, the mushroom mycelium used yeast extracts as nitrogen sources in liquid medium, such as *Lyophyllum decastes* (Pokhrel and Ohga 2007a), *Cordyceps jiangxiensis* (Xiao *et al.* 2006), *Lentinus subnudus* (Gbolagade *et al.* 2006), *Lentinus squarrosulus* (Das *et al.* 2015), and *Pleurotus albidus* (Kirsch *et al.* 2016). This is mainly because yeast extract is rich in protein, amino acids, and vitamins, which contributes to cell growth.



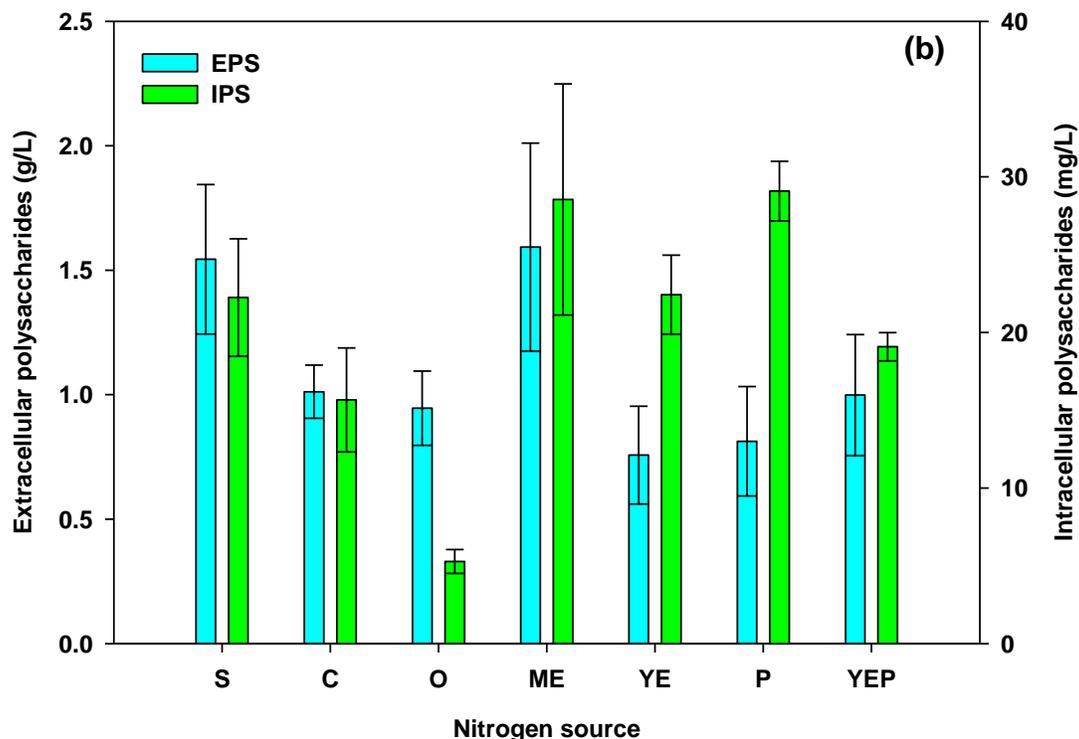


Fig. 1. Effect of single and dual nitrogen sources for the production of mycelia biomass by *G. frondosa* on the final pH of the medium (a) extracellular polysaccharides (EPS), and intracellular polysaccharides (IPS) (b); Nitrogen sources: S, ammonium sulfate; C, ammonium chloride; O, ammonium oxalate; ME, malt extract; YE, yeast extract; P, peptone; YEP, yeast extract plus peptone

In this study, the mycelial biomass (1.98 g/L) of *G. frondosa* in dual nitrogen sources was not higher in this study than that of yeast extracts. However, some previous studies have suggested the use of dual nitrogen sources to promote the production of mycelium biomass. Fang and Zhong (2002) found that a single nitrogen source resulted in a lower mycelia yield, and mixed yeast extracts and peptone were more conducive for the growth of *Ganoderma lucidum* cultures. Dang *et al.* (2018) also indicated that using an optimal ratio of yeast extract and skim milk for the nitrogen source can produce high amounts of mycelial biomass. In a study by Shih *et al.* (2008), dual nitrogen sources of *G. frondosa* were used. Considering the earlier reports and the results of this study, different combinations and proportions of dual nitrogen sources may affect mycelial production. When a composition of non-nitrogen sources is added to a single source of nitrogen, mycelial growth is enhanced, which indicates a key starting point for future studies.

The EPS production amounts with various nitrogen sources ranged from 0.75 g/L to 1.58 g/L. Malt extract and ammonium sulfate were the best nitrogen sources for the production of EPS at 1.58 g/L and 1.54 g/L, respectively, and their influence on EPS production did not differ significantly. The EPS production in the medium that contained ammonium chloride and ammonium oxalate was higher than that using yeast extracts and peptone. A similar result was reported on *Lyophyllum decastes* (Pokhrel and Ohga 2007a; Lin 2011; Anike *et al.* 2015); in particular, a correlation between mycelia growth and EPS

production was observed. Thus, EPS production was reduced with maximum mycelial growth. Conversely, with minimum mycelial growth, high EPS production was observed.

Figure 1 shows that, utilizing various sources of nitrogen, IPS production ranged from 5.3 mg/L to 29.1 mg/L. The best nitrogen source for the production of IPS was observed to be peptone (29.1 mg/L), followed by yeast extract (22.4 mg/L), and ammonium sulfate (22.2 mg/L). For EPS (g/L) and IPS (mg/L) production, the second-best source of nitrogen was ammonium sulfate. Moreover, malt extract was more productive for EPS and IPS than yeast extract. These results indicated that inorganic nitrogen sources appeared to positively affect polysaccharide production, while utilizing a specific nitrogen source. Such a phenomenon was also reported in submerged cultures of *Lentinus squarrosulus* (Ahmad *et al.* 2013).

Distribution Percentage of Biomass (DPB) in Fractions L, S, and A

Organic, inorganic, and dual nitrogen sources were compared in terms of L, S, and A fractions in the fermentation broth, and the size distribution percentage of the total biomass was calculated (Fig. 2). Among the various nitrogen sources, yeast extract yielded the highest mycelial growth, and the proportions of the L, S, and A fractions were 47%, 20%, and 33%, respectively. Similarly, in mycelial biomass production cultured with dual nitrogen sources (YEP) of the second-best DPB for (Fig. 2), fractions L, S, and A were 39%, 7%, and 53%, respectively. This result was calculated in terms of dry weight, which was mainly provided by large pellets.

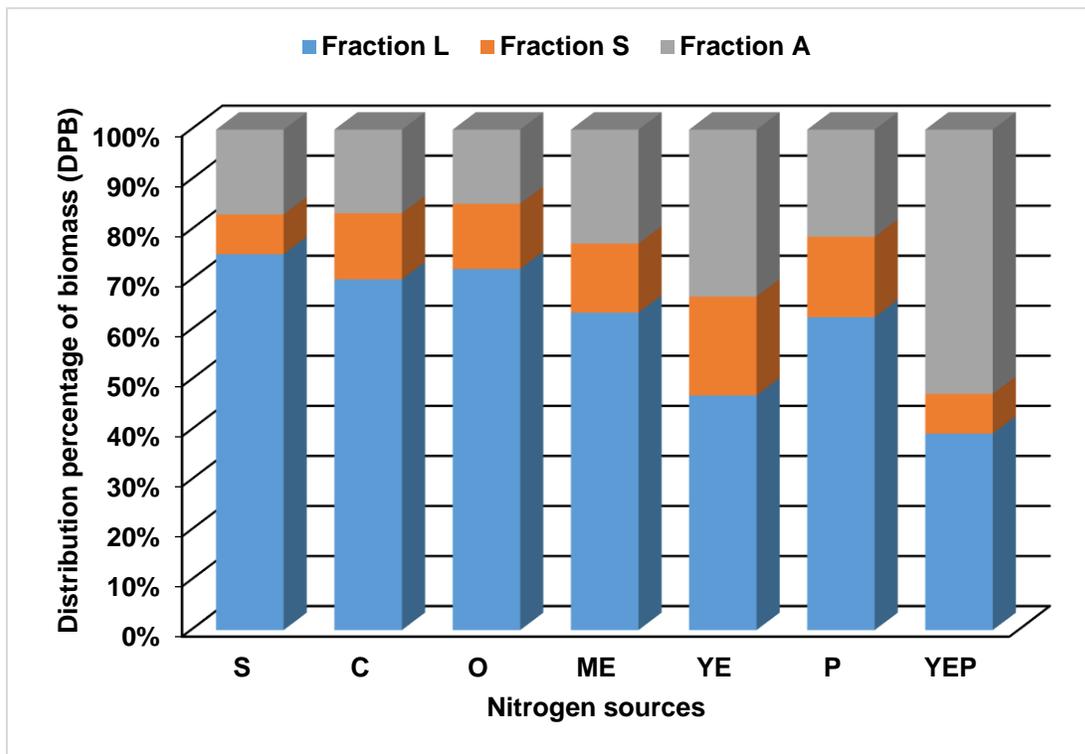


Fig. 2. Effect of nitrogen sources on the DPB in terms of pellet size and adhesive mycelium in fractions L, S, and A in cultures of *G. frondosa*. Nitrogen sources: S, ammonium sulfate; C, ammonium chloride; O, ammonium oxalate; ME, malt extract; YE, yeast extract; P, peptone; YEP, yeast extract plus peptone

The distribution ratio of the fractions was revealed by comparing the sizes of the pellets in the fermentation broth. The lower percentage of fraction A and the higher percentage of fraction S in the media broth indicated an increase in mycelial production. These data clearly showed that the morphological change was affected by the metabolic activity, and an optimal morphology of the pellets was maintained, which resulted in a high yield of metabolites. These results were consistent with those previously reported for *Aspergillus niger* (Hille *et al.* 2005), *G. lucidum* (Fang and Zhong 2002), and *Grifola frondosa* (Tao *et al.* 2018).

Distribution Percentage of Intracellular Polysaccharides (DPI) in Fractions L, S, and A

Pellet morphology has been cited as a key determinant of fermentation productivity. In this study, individual IPS production levels in fractions L, S, and A were determined to understand the different mycelial pellet types for IPS production. The supply percentages for fractions L, S, and A were calculated with Eq. 2:

$$\text{Individual IPS / Total amount of IPS} \times 100\% \quad (2)$$

The supply percentage of IPS production using peptone culture consisted of 71% fraction L, 11% fraction S, and 16% fraction A, as shown in Fig. 3.

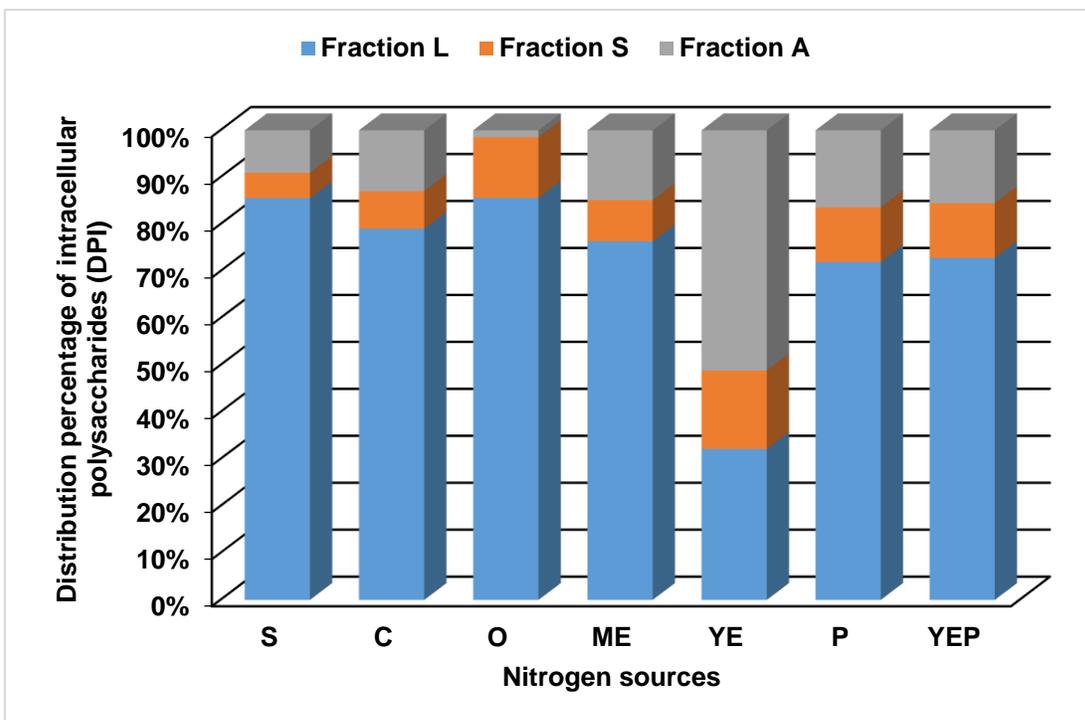


Fig. 3. Effect of various nitrogen sources on the distribution percentage of intracellular polysaccharides in terms of pellet size and adhesive mycelium in fraction L, S, and A in the culture of *G. frondosa*. Nitrogen sources: S, ammonium sulfate; C, ammonium chloride; O, ammonium oxalate; ME, malt extract; YE, yeast extract; P, peptone; YEP, yeast extract plus peptone

The second highest productivity of polysaccharides was observed in malt extract cultures, but the IPS supply percentage by fraction L accounted for 76%, and fractions S and A accounted for 8%, and 14%, respectively. These results revealed that the optimum

IPS production and accumulation was the cause for the morphology of the large pellets (fraction L), whereas the hyphae formed by smaller pellets (fraction S) and the adhesive hyphae on the sides of the bottle (fraction A) were unable to accumulate IPS. A relationship between pellet morphology and metabolite production was also reported by Papagianni and Murray (2002) and Chen *et al.* (2016).

Initial NH₄⁺ Concentration and NH₄⁺ Utilization

Several earlier studies have reported that organic nitrogen increases mycelium biomass and its metabolites (Lee *et al.* 2004; Xu *et al.* 2008; Wen *et al.* 2014; Salami *et al.* 2017). Likewise, in this study, higher biomass production due to yeast extract and dual nitrogen sources (Fig. 1) was observed. In particular, ammonium sulfate yielded the third-highest amount of inorganic nitrogen. This may have been due to a positive correlation between the concentration of NH₄⁺ in the medium and the extent of nitrogen utilization.

Using a Kjeldahl analysis, it was observed that initial NH₄⁺ concentrations over 2.01 g/L and less than 0.29 g/L were not suitable for mycelial growth (Table 1). Similar results were observed in a study on *Cordyceps militaris*, in which the medium that contained 10 mM NH₄⁺ was optimal for mycelial growth. Further, high growth inhibition (low growth) was observed in 10 mM NH₄⁺ (Mao and Zhong 2006). In addition, the initial NH₄⁺ concentration from organic nitrogen was noticeably low, and the results showed that other substances (*i.e.*, amino acids, minerals, and vitamins) may also be important for cell growth in addition to NH₄⁺. When the nitrogen sources were favorable for mycelial growth, NH₄⁺ utilization was over 97%. Thus, the levels of NH₄⁺ affected the utilization of growth factors in the production of mycelia. This was consistent with the NH₄⁺ content in peptone, which was exhausted; then, a different NH₄⁺ source (ammonium sulfate) was fed for cordycepin production in *C. militaris* (Miqueleto *et al.* 2010).

Table 1. Initial NH₄⁺ Concentration and NH₄⁺ Utilization from Nitrogen Sources in Media by *Grifola frondosa*

Nitrogen Sources in Medium	Ammonium Cation (NH ₄ ⁺)		
	Original (g/L)	Residue (g/L)	Utilization Rate (%) [*]
Ammonium sulfate	1.58	0.023	98.5
Ammonium chloride	2.01	0.799	60.7
Ammonium oxalate	3.12	0.552	72.7
Malt extract	0.29	0.088	69.7
Yeast extract	0.65	0.005	99.2
Peptone	0.98	0.027	97.2
YEP**	0.70	0.012	98.3

* Utilization rate = (Depletion of NH₄⁺ / Original of NH₄⁺) × 100%
 ** YEP = yeast extract plus peptone

Morphological Parameters

The morphological properties of filamentous fungus play an important role in their metabolism during fermentation (Moreira *et al.* 1996; Nielsen 1996; Wan-Mohtar *et al.* 2016). Accordingly, estimates of the compactness, circularity, roughness, and the total

number of pellets in submerged cultures of *G. frondosa* using different nitrogen sources are shown in Table 2. There was remarkable variation in the morphological parameters between the pellets grown under organic and inorganic nitrogen sources. The maximum circularity and minimum roughness values were observed when the yeast extract was used as the nitrogen source for mycelial biomass production. Similar results were obtained after using both ammonium sulfate and a dual source of nitrogen. In addition, the maximum compactness value and the minimum total number of pellets in the broth were desirable for biomass production. To obtain a smaller diameter, the hairy hyphae were sheared to form denser circular pellets, which increased the productive morphological forms and mycelium biomass production by *G. frondosa*.

Table 2. Compactness, Circularity, Roughness, and Total Number of Pellets of *Grifola frondosa* Grown Using Seven Different Nitrogen Sources

Nitrogen Sources in Medium	Pellets			
	Compactness	Circularity	Roughness	Total Number
Ammonium sulfate	0.96	0.36	1.64	48
Ammonium chloride	0.69	0.18	2.46	120
Ammonium oxalate	0.69	0.19	2.43	102
Malt extract	0.53	0.15	2.86	73
Yeast extract	0.85	0.63	1.24	23
Peptone	0.69	0.27	1.98	77
YEP*	0.60	0.44	1.69	253

*YEP = yeast extract plus peptone

Next, the association between extracellular polysaccharides with the imaging parameters was examined. As shown in Fig. 1a, both the compactness (0.53) and circularity (0.15) of the pellets were low in 7 types of nitrogen sources, but pellet roughness was the highest when malt extract was used, which was the nitrogen source that resulted in maximum polysaccharide production. A pellet morphology with high hairiness, fluff hyphae, and a small core area in media was observed for EPS production with nitrogen from ammonium chloride, and other similar morphologies were observed. During fermentation, the outer hairy region of the pellets was shaved off because shaved hyphae are a function of hydrodynamic forces. The new pellets were developed from hyphal fragments. This phenomenon indicated an increase in the total number of pellets, which enhanced the yield of the mycelial biomass. Further, pellet autolysis was accelerated using specific nitrogen sources, which led to the formation of fewer mycelial pellets. In addition, the IPS was released into the fermentation broth and enhanced the EPS yield.

Fungal Morphology

Medium composition has been recognized as a strong determinant of fungal morphology, and both the type of nitrogen source and concentration are particularly influential. As shown in Fig. 4, the typical fungal morphologies, which were assessed using the Stereo microscope and an ion-sputtering device, were dependent on different nitrogen sources.

A smooth pellet growth with a less hairy region (Fig. 4e) and hyphae relative to cross-linkages (Fig. 5e), resulted in fewer pellets (Table 2), and a high rate of adhesive hyphae formed on the sides of the bottle (Fig. 2) using the yeast extract. Therefore, it was concluded that the hyphae began to detach from the original pellets. These detached fragments adhered to the bottle. The hyphae were easily broken, and the re-formation of

pellets was difficult, which caused stacking. Intermittent control of the fluid shear stress to maintain a particular hyphae form may increase the mycelium biomass (Park *et al.* 2002a; Cui *et al.* 2016).

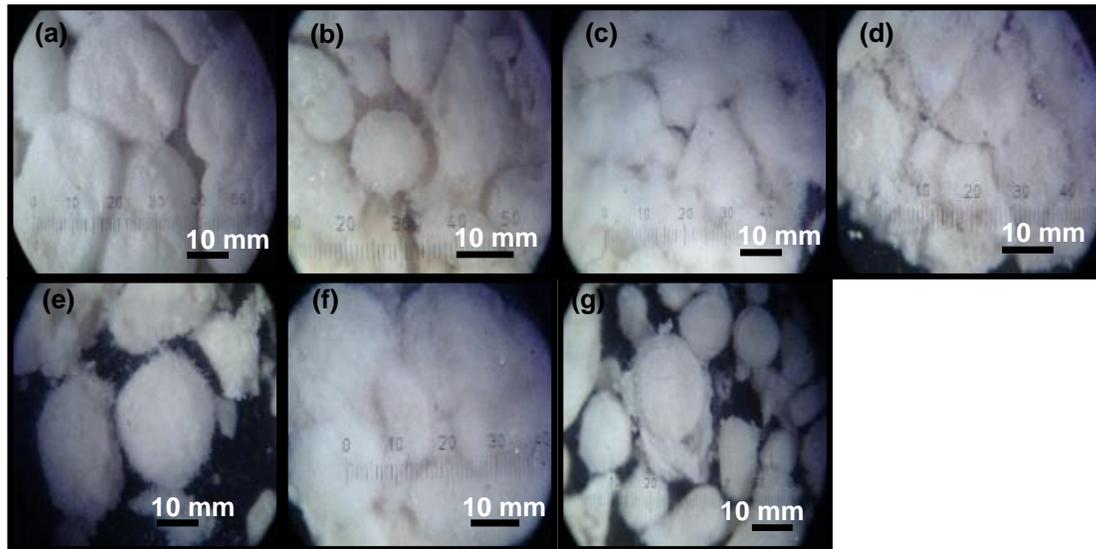


Fig. 4. Typical morphologies of *G. frondosa* after using different nitrogen sources assessed using a stereo microscope. Nitrogen sources: (a) ammonium sulfate; (b) ammonium chloride; (c) ammonium oxalate; (d) malt extract; (e) yeast extract; (f) peptone; (g) yeast extract plus peptone

In cultures using malt extract, the freeze-drying treatment of the pellets resulted in aggregation (Fig. 4d) and peak roughness (Table 2). Pellets with high hairiness resulted in aggregation with reduced fluid shear stress. The intermingling of hyphae from the surface was not compact (Fig. 5d). Such conditions may have resulted in easier access to the pellet center *via* the broth and cause the release of EPS to the fermentation broth. An obvious pellet formation was demonstrated using peptone (Fig. 4f) and malt extract (Fig. 4d).

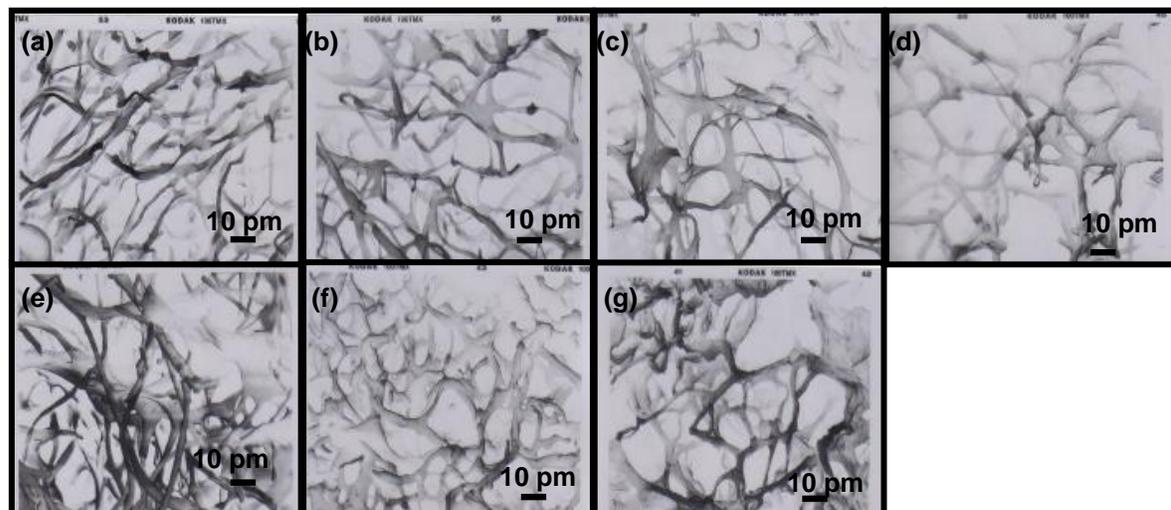


Fig. 5. Typical morphologies of pellets formed using different nitrogen sources and examined through the ion sputtering device. Nitrogen sources: (a) ammonium sulfate; (b) ammonium chloride; (c) ammonium oxalate; (d) malt extract; (e) yeast extract; (f) peptone; (g) yeast extract plus peptone

The surface of the mycelial hyphae was thick (Fig. 5d and Fig. 5f), and the pattern may have helped to protect the mycelium and facilitate the accumulation of IPS in the hyphal body.

CONCLUSIONS

1. The fungus *G. frondosa* was effectively grown in submerged cultures for the production of both extracellular polysaccharides (EPS) and intracellular polysaccharides (IPS).
2. Yeast extract is beneficial to the formation of mycelial biomass, and malt extract and ammonium sulfate were the best sources of nitrogen for EPS production, whereas peptone was preferred for high IPS production. However, higher NH_4^+ utilization (up to 97%) resulted in the production of mycelial biomass; thus, the stimulatory effect of NH_4^+ on metabolic biosynthesis requires further investigation.
3. Knowledge of the growth physiology of *G. frondosa* remains limited. It is still unclear why the morphological change varies depending on the nitrogen source used. From a morphological perspective, maximum circularity value and minimum roughness were desirable for mycelial biomass production. In addition, the minimum compactness value was desirable for mycelial biomass production, and production of IPS resulted in large mycelial pellets.
4. The results of this study have identified several phenomena and opened avenues for future studies, particularly those investigating the underlying mechanism linking pellet morphology with metabolic activities. However, further studies are required to improve the production of the mycelia and polysaccharides of *G. frondosa*.

REFERENCES CITED

- Ahamed, A., and Vermette, P. (2009). "Effect of culture medium composition on *Trichoderma reesei*'s morphology and cellulase production," *Bioresour. Technol.* 100(23), 5979-87.
- Ahmad, R., Al-Shorgani, N. K. N., Hamid, A. A., Yusoff, W. M.W., and Daud, F. (2013). "Optimization of medium components using response surface methodology (RSM) for mycelium biomass and exopolysaccharide production by *Lentinus squarrosulus*," *Adv. Biosci. Biotechnol.* 4(12), 1079-1085.
- Ahumada-Rudolph, R., Novoa, V., and Becerra, J. (2019). "Morphological response to salinity, temperature, and pH changes by marine fungus *Epicoccum nigrum*," *Environ. Monit. Assess.* 191, 35.
- Allen, S. E., Grimshaw, H. M., Parkinson, J. A., Quarmby, C., and Roberts, J. D. (1974). *Chemical Analysis of Ecological Materials*, John Wiley & Sons, New York, NY, USA.
- Anike, F., Isikhuemhen, O. S., Blum, D., and Neda, H. (2015). "Nutrient requirements and fermentation conditions for mycelia and crude exo-polysaccharides production by *Lentinus squarrosulus*," *Adv. Biosci. Biotechnol.* 6(8), 526-536.

- Antecka, A., Bizukojc, M., and Ledakowicz, S. (2016). "Modern morphological engineering techniques for improving productivity of filamentous fungi in submerged cultures," *World J. Microbiol. Biotechnol.* 32(12), 193.
- Chen, W., Zhao, Z., Chen, S.-F., and Li, Y.-Q. (2008). "Optimization for the production of exopolysaccharide from *Fomes fomentarius* in submerged culture and its antitumor effect *in vitro*," *Bioresource Technol.* 99(8), 3187-3194. DOI: 10.1016/j.biortech.2007.05.049
- Chen, X. X., Yang, Y., Cui, F. J., Sun, W. J., and Liu, W. M. (2016). "Effect of aluminum oxide on *Grifola frondosa* mycelial biomass, pellet diameter and polysaccharide production," *Acta Edible Fungi* 23(1), 42-6.
- Cui, F.-J., Li, Y., Xu, Y.-Y., Liu, Z.-Q., Huang, D.-M., Zhang, Z.-C., and Tao, W.-Y. (2007). "Induction of apoptosis in SGC-7901 cells by polysaccharide-peptide GFPS1b from the cultured mycelia of *Grifola frondosa* GF9801," *Toxicol. in Vitro* 21(3), 417-427. DOI: 10.1016/j.tiv.2006.10.004
- Cui, F. J., Li, Y., Xu, Z. H., Xu, H. Y., Sun, K., and Tao, W. Y. (2006). "Optimization of the medium composition for production of mycelial biomass and *exo*-polymer by *Grifola frondosa* GF9801 using response surface methodology," *Bioresource Technol.* 97(10), 1209-1216. DOI: 10.1016/j.biortech.2005.05.005
- Cui, F. J., Chen, X. X., Liu, W. M., Sun, W. J., Huo, S. H., and Yang, Y. (2016). "Control of *Grifola frondosa* morphology by agitation and aeration for improving mycelia and *exo*-polymer production," *Appl. Biochem. Biotechnol.* 179(3), 459-473.
- Dang, H. N., Wang, C. L., and Lay, H. L. (2018). "Effect of nutrition, vitamin, grains, and temperature on the mycelium growth and antioxidant capacity of *Cordyceps militaris* (strains AG-1 and PSJ-1)," *J. Radiat. Res. Appl. Sci.* 11, 130-138.
- Das, A. R., Borthakur, M., Saha, A. K., Joshi, S. R., and Das, P. (2015). "Growth of mycelial biomass and fruit body cultivation of *Lentinus squarrosulus* collected from home garden of Tripura in Northeast India," *J. App. Biol. Biotech.* 3(4), 17-19.
- Ding, Y. R., Xiao, C., Wu, Q. P., Xie, Y. Z., Li, X. M., Hu, H. P., and Li, L. Q. (2016). "The mechanisms underlying the hypolipidaemic effects of *Grifola frondosa* in the liver of rats," *Front. Microbiol.* 3(7), 1186.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956). "Colorimetric method for determination of sugars and related substances," *Anal. Chem.* 28(3), 350-356. DOI: 10.1021/ac60111a017
- Fang, Q.-H., and Zhong, J.-J. (2002). "Two-stage culture process for improved production of ganoderic acid by liquid fermentation of higher fungus *Ganoderma lucidum*," *Biotechnol. Prog.* 18(1), 51-54. DOI: 10.1021/bp010136g
- Gbolagade, J. S., Fasidi, I. O., Ajayi, E. J., and Sobowale A. A. (2006). "Effect of physico-chemical factors and semi-synthetic media on vegetative growth of *Lentinus subnudus* (Berk.), an edible mushroom from Nigeria," *Food Chem.* 99(4), 742-747. DOI: 10.1016/j.foodchem.2005.08.052
- Gu, C.-Q., Li, J.-W., Chao, F., Jin, M., Wang, X.-W., and Shen, Z.-Q. (2007). "Isolation, identification and function of a novel anti-HSV-1 protein from *Grifola frondosa*," *Antiviral Res.* 75(3), 250-257. DOI: 10.1016/j.antiviral.2007.03.011
- Hille, A., Neu, T. R., Hempel, D. C., and Horn, H. (2005). "Oxygen profiles and biomass distribution in biopellets of *Aspergillus niger*," *Biotechnol. Bioeng.* 92(5), 614-623. DOI: 10.1002/bit.20628
- Hsieh, C., Liu, C.-J., Tseng, M.-H., Lo, C.-T., and Yang, Y.-C. (2006). "Effect of olive oil on the production of mycelial biomass and polysaccharides of *Grifola frondosa*

- under high oxygen concentration aeration,” *Enzyme Microb. Tech.* 39(3), 434-439. DOI: 10.1016/j.enzmictec.2005.11.033
- Huang, H.-C., and Liu, Y.-C. (2007). “Acceleration of mycelia growth by adding *Grifola umbellata* broth concentrate in solid-state and submerged cultures,” *Biochem. Eng. J.* 37(2), 139-143. DOI: 10.1016/j.bej.2007.04.003
- Kim, S.-W., Hwang, H.-J., Xu, C.-P., Sung, J.-M., Choi, J.-W., and Yun, J.-W. (2003). “Optimization of submerged culture process for the production of mycelial biomass and exo-polysaccharides by *Cordyceps militaris* C738,” *J. Appl. Microbiol.* 94(1), 120-126. DOI: 10.1046/j.1365-2672.2003.01754.x
- Kirsch, L. S., de Macedo, A. J. P., and Teixeira, M. F. S. (2016). “Production of mycelial biomass by the Amazonian edible mushroom *Pleurotus albidus*,” *Braz. J. Microbiol.* 47(3), 658-64.
- Kodama, N., Murata, Y., Asakawa, A., Inui, A., Hayashi, M., Sakai, N., and Nanba, H. (2005). “Maitake D-fraction enhances antitumor effects and reduces immunosuppression by mitomycin-C in tumor-bearing mice,” *Nutrition* 21(5), 624-629. DOI: 10.1016/j.nut.2004.09.021
- Kubo, K., and Nanba, H. (1997). “Anti-hyperliposis effect of maitake fruit body (*Grifola frondosa*). I,” *Biol. Pharm. Bull.* 20(7), 781-785. DOI: 10.1248/bpb.20.781
- Lee, B. C., Bae, J. T., Pyo, H. B., Choe, T. B., Kim, S. W., Hwang H. J., and Yun, J. W. (2004). “Submerged culture conditions for the production of mycelial biomass and exopolysaccharides by the edible Basidiomycete *Grifola frondosa*,” *Enzyme Microb. Tech.* 35(5-6), 369-376. DOI: 10.1016/j.enzmictec.2003.12.015
- Lee, J.-S., Park, B. C., Ko, Y. J., Choi, M. K., Choi, H. G., Yong, C. S., Lee, J.-S., and Kim, J.-A. (2008). “*Grifola frondosa* (maitake mushroom) water extract inhibits vascular endothelial growth factor-induced angiogenesis through inhibition of reactive oxygen species and extracellular signal-regulated kinase phosphorylation,” *J. Med. Food* 11(4), 643-651. DOI: 10.1089/jmf.2007.0629
- Lin, E. S. (2011). “Production of exopolysaccharides by submerged mycelial culture of *Grifola frondosa* TFR11073 and their antioxidant and antiproliferative activities,” *World J. Microbiol. Biotechnol.* 27(3), 555-561.
- Lo, H.-C., Hsu, T.-H., and Chen, C.-Y. (2008). “Submerged culture mycelium and broth of *Grifola frondosa* improve glycemic responses in diabetic rats,” *Am. J. Chinese Med.* 36(2), 265-285. DOI: 10.1142/S0192415X0800576X
- Li, L., Guo, W. L., Zhang, W., Xu, J. X., Qian, M., Bai, W. D., Zhang, Y. Y., Rao, P. F., Ni, L., and Lv, X. C. (2019). “*Grifola frondosa* polysaccharides ameliorate lipid metabolic disorders and gut microbiota dysbiosis in high-fat diet fed rats,” *Food Funct.* 10(5), 2560-2572.
- Mao, X.-B., and Zhong, J.-J. (2006). “Significant effect of NH_4^+ on cordycepin production by submerged cultivation of medicinal mushroom *Cordyceps militaris*,” *Enzyme Microb. Tech.* 38(3-4), 343-350. DOI: 10.1016/j.enzmictec.2004.10.010
- Mao, G. H., Ren, Y., Feng, W. W., Li, Q., Wu, H. Y., Jin, D., Zhao, T., Xu, C. Q., Yang, L. Q., and Wu, X. Y. (2015). “Antitumor and immunomodulatory activity of a water-soluble polysaccharide from *Grifola frondosa*,” *Carbohydr. Polym.* 10, 134:406-12.
- Masuda, Y., Inoue, M., Miyata, A., Mizuno, S., and Nanba, H. (2009a). “Maitake beta-glucan enhances therapeutic effect and reduces myelosuppression and nephrotoxicity of cisplatin in mice,” *Int. Immunopharmacol.* 9(5), 620-626. DOI: 10.1016/j.intimp.2009.02.005

- Masuda, Y., Matsumoto, A., Toida, T., Oikawa, T., Ito, K., and Nanba, H. (2009b). "Characterization and antitumor effect of a novel polysaccharide from *Grifola frondosa*," *J. Agr. Food Chem.* 57(21), 10143-10149. DOI: 10.1021/jf9021338
- Miqueleto, A. P., Dolosic, C. C., Pozzi, E., Foresti, E., and Zaiat, M. (2010). "Influence of carbon sources and C/N ratio on EPS production in anaerobic sequencing batch biofilm reactors for wastewater treatment," *Bioresour. Technol.* 101(4), 1324–1330.
- Mizuno, T., and Zhuang, C. (1995). "Maitake, *Grifola frondosa*: Pharmacological effects," *Food Rev. Int.* 11(1), 135-149. DOI: 10.1080/87559129509541024
- Moreira, M. T., Feijoo, G., Sanromán, A., and Lema, J. M. (1996). "Effect of pulsation on morphology of *Aspergillus niger* and *Phanerochaete chrysosporium* in a fluidized-bed reactor," *Progr. Biotechnol.* 11, 518-523. DOI: 10.1016/S0921-0423(96)80071-2
- Mori, K., Kobayashi, C., Tomita, T., Inatomi, S., and Ikeda, M. (2008). "Antiatherosclerotic effect of the edible mushrooms *Pleurotus eryngii* (Eringi), *Grifola frondosa* (Maitake), and *Hypsizygus marmoreus* (Bunashimeji) in apolipoprotein E-deficient mice," *Nutr. Res.* 28(5), 335-342. DOI: 10.1016/j.nutres.2008.03.010
- Nanba, H., Kodama, N., Schar, D., and Turner, D. (2000). "Effects of maitake (*Grifola frondosa*) glucan in HIV-infected patients," *Mycoscience* 41(4), 293-295. DOI: 10.1007/BF02463941
- Nie, X., Shi, B., Ding, Y., and Tao, W. (2006). "Preparation of a chemically sulfated polysaccharide derived from *Grifola frondosa* and its potential biological activities," *Int. J. Biol. Macromol.* 39(4-5), 228-233. DOI: 10.1016/j.ijbiomac.2006.03.030
- Nielsen, J. (1996). "Modelling the morphology of filamentous microorganisms," *Trends Biotechnol.* 14(11), 438-443. DOI: 10.1016/0167-7799(96)10055-X
- Papagianni, M., and Murray, M. Y. (2002). "Protease secretion in glucoamylase producer *Aspergillus niger* cultures: Fungal morphology and inoculum effects," *Process Biochem.* 37(11), 1271-1278. DOI: 10.1016/S0032-9592(02)00002-X
- Park, E. Y., Koike, Y., Higashiyama, K., Fujikawa, S., and Okabe, M. (1999). "Effect of nitrogen source on mycelial morphology and arachidonic acid production in cultures of *mortierella alpina*," *J. Biosci. Bioeng.* 88(1), 61-67. DOI: 10.1016/S1389-1723(99)80177-7
- Park, J. P., Kim, Y. M., Kim, S. W., Hwang, H.-J., Cho, Y. J., Lee, Y. S., Song, C. H., and Yun, J. W. (2002a). "Effect of aeration rate on the mycelial morphology and *exo*-biopolymer production in *Cordyceps militaris*," *Process Biochem.* 37(11), 1257-1262. DOI: 10.1016/S0032-9592(02)00005-5
- Park, J.-P., Kim, S.-W., Hwang, H.-J., Cho, Y.-J., and Yun, J.-W. (2002b). "Stimulatory effect of plant oils and fatty acids on the *exo*-biopolymer production in *Cordyceps militaris*," *Enzyme Microb. Tech.* 31(3), 250-255. DOI: 10.1016/S0141-0229(02)00099-6
- Paul, G. C., Priede, M. A., and Thomas, C. R. (1999). "Relationship between morphology and citric acid production in submerged *Aspergillus niger* fermentations," *Biochem. Eng. J.* 3(2), 121-129. DOI: 10.1016/S1369-703X(99)00012-1
- Pokhrel, C. P., and Ohga, S. (2007a). "Synthetic cultivation of *Lyophyllum decastes* on a combination of livestock compost and corn cobs," *Mushroom Sci. Biotech.* 15(3), 123-128. DOI: 10.24465/msb.15.3_123
- Preuss, H. G., Echard, B., Bagchi, D., Perricone, N. V., and Zhuang, C. (2007). "Enhanced insulin-hypoglycemic activity in rats consuming a specific glycoprotein

- extracted from maitake mushroom,” *Mol. Cell. Biochem.* 306(1-2), 105-113. DOI: 10.1007/s11010-007-9559-6
- Riley, G. L., Tucker, K. G., Paul, G. C., and Thomas, C. R. (2000). “Effect of biomass concentration and mycelial morphology on fermentation broth rheology,” *Biotechnol. Bioeng.* 68(2), 160-172. DOI: 10.1002/(SICI)1097-0290(20000420)68:2<160:AID-BIT5>3.0.CO;2-P
- Salami, A. O., Bankole, F. A., and Andrew, O. C. (2017). “Effect of organic nitrogen supplements on the yield and nutrient content of oyster mushroom (*Pleurotus florida*) cultivated on corncobs,” *SF J Mycology* 1, 1.
- Shi, B. J., Nie, X.-H., Chen, L.-Z., Liu, Y.-L., and Tao, W.-Y. (2007). “Anticancer activities of a chemically sulfated polysaccharide obtained from *Grifola frondosa* and its combination with 5-fluorouracil against human gastric carcinoma cells,” *Carbohydr. Polym.* 68(4), 687-692. DOI: 10.1016/j.carbpol.2006.08.003
- Shih, I.-L., Chou, B.-W., Chen, C. C., Wu, J.-Y., and Hsieh, C. (2008). “Study of mycelial growth and bioactive polysaccharide production in batch and fed-batch culture of *Grifola frondosa*,” *Bioresource Technol.* 99(4), 785-793. DOI: 10.1016/j.biortech.2007.01.030
- Tao, T. L., Cui, F. J., Chen, X. X., Sun, W. J., Huang, D. M., Zhang, J. S., Yang, Y., Wu, D., and Liu, W. M. (2018). “Improved mycelia and polysaccharide production of *Grifola frondosa* by controlling morphology with microparticle talc,” *Microb. Cell Fact.* 17, 1.
- Veiter, L., Rajamanickam, V., and Herwig, C. (2018). “The filamentous fungal pellet—relationship between morphology and productivity,” *Appl. Microbiol. Biotechnol.* 102, 2997-3006.
- Wagner, R., Mitchell, D. A., Sasaki, G. L., and de Almeida Amazonas, M. A. L. (2004). “Links between morphology and physiology of *Ganoderma lucidum* in submerged culture for the production of exopolysaccharide,” *J. Biotechnol.* 114(1-2), 153-164. DOI: 10.1016/j.jbiotec.2004.06.013
- Wang, Y., Xie, S., Sun, T., Zhang, D., Sun, Q., Sun, Y., Feng, Y., and Xin, H. (2010). “Inhibitory effect of the polysaccharide of *Grifola frondosa* on carbon tetrachloride induced injury to the liver cell line L02,” *J. Shandong Univ. (Health Sci.)* 48(8), 32-37.
- Wan-Mohtar, W. A. A. Q. I., Kadir, S. A., and Saari, N. (2016). “The morphology of *Ganoderma lucidum* mycelium in a repeated-batch fermentation for exopolysaccharide production,” *Biotechnol. Rep.* 11, 2-11.
- Wen, T. C., Li, G. R., Kang, J. C., Kang, C., and Hyde, K. D. (2014). “Optimization of solid-state fermentation for fruiting body growth and cordycepin production by *Cordyceps militaris*,” *Chiang Mai J. Sci.* 41(4), 858-872.
- Wu, T. X., Wang, N., Zhang, Y., and Xu, X. B. (2016). “Research progress in submerged mycelial culture of *Grifola frondosa*, a culinary-medicinal mushroom,” *Afr. J. Microbiol. Res.* 10(6), 138-146.
- Wu, J.-Y., Siu, K.-C., and Geng, P. (2021). “Bioactive ingredients and medicinal values of *Grifola frondosa* (maitake),” *Foods* 10(1), 95.
- Xiao, J.-H., Chen, D.-X. Wan, W.-H., Hu, X.-J., Qi, Y., and Liang, Z. Q. (2006). “Enhanced simultaneous production of mycelia and intracellular polysaccharide in submerged cultivation of *Cordyceps jiangxiensis* using desirability functions,” *Process Biochem.* 41(8), 1887-1893. DOI: 10.1016/j.procbio.2006.03.031

Xu, P., Ding, Z.-Y., Qian, Z., Zhao, C.-X., and Zhang, K.-C. (2008). "Improved production of mycelial biomass and ganoderic acid by submerged culture of *Ganoderma lucidum* SB97 using complex media," *Enzyme Microb. Tech.* 42(4), 325-331. DOI: 10.1016/j.enzmictec.2007.10.016

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