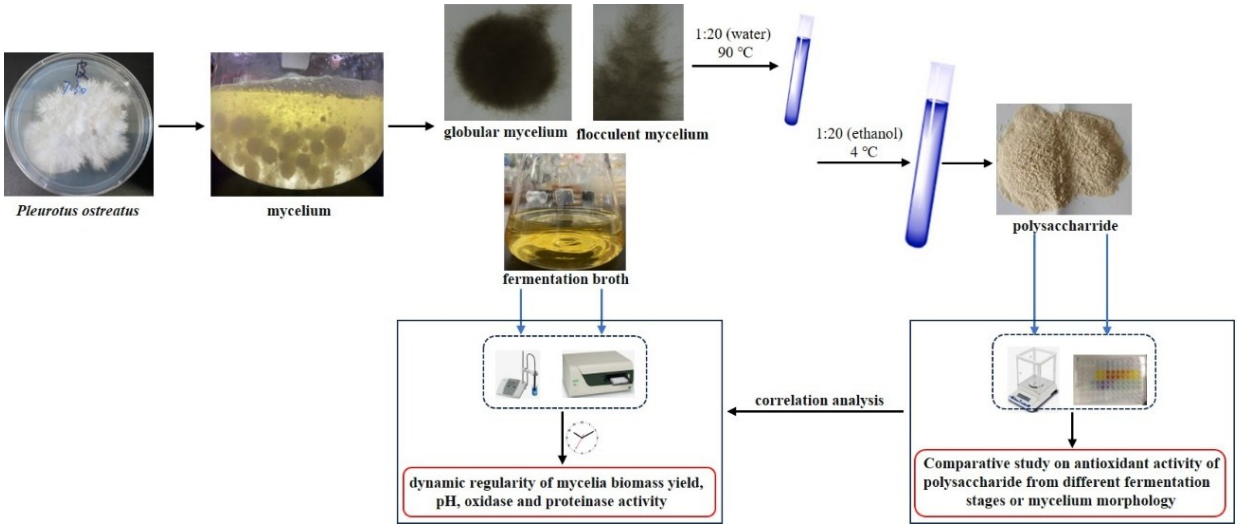
Effect of Mycelium Morphology on Fermentation Productivity and Antioxidant Activity of Polysaccharide from *Pleurotus ostreatus*

Libing Zheng,[a] Li Wang,[a] Yuexin Li,[a,*] Xuanmei Tong,[a] and Xiangyun Zheng [b,*]

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GRAPHICAL ABSTRACT



Effect of Mycelium Morphology on Fermentation Productivity and Antioxidant Activity of Polysaccharide from *Pleurotus ostreatus*

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Globular or clumped mycelium of *Pleurotus ostreatus* appeared in different batches during the fermentation process. A 10-days fermentation was carried out. Globular mycelium in one batch with relatively uniform particle size (4.08±1.28 mm) could produce 11.10 g/L biomass, 2.19 mg glucose equivalent /L water-soluble crude endo-polysaccharides (*Po*-IPS), and 4.33 mg glucose equivalent /L exopolysaccharides (*Po*-EPS), which were higher than globular mycelium with significantly different pellet size (10.38±7.65 mm) in the other batch. The maximum biomass production of clumped mycelium was 9.81g/L at 2d with 15% inoculum volume, while it was 10.27g/L at 4d with 7.5% inoculum volume, suggesting inoculum volume significantly affected fermentation process. *Po*-IPS production of globular mycelium was 1.5-folds higher than that of clumped mycelium, while *Po*-EPS production of clumped mycelium was 2.8-folds higher than that from globular mycelium. Surprisingly, the scavenging rate on OH· (>80%) or DPPH (>70%) radicals of crude *Po*-IPS from globular mycelium was 10-folds, 7-folds higher than that from clumped mycelium, respectively. Total antioxidant capacity also presented a similar trend. During the fermentation of globular mycelium, pH 6.4 was the optimal, laccase was the main one, lignin peroxidase, manganese peroxidase, and other decomposition enzyme helped to decompose the starch. The data demonstrated that mycelial morphology of *P. ostreatus* affected fermentation output, and functional activity of the polysaccharide.

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Keywords: Pleurotus ostreatus; Mycelial morphology; Submerged fermentation; Polysaccharide; Antioxidant activity

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INTRODUCTION

Edible and medicinal fungi have been an important dietary component from ancient times. Because of their high yield, easy cultivation, and rich bioactive substances, they are widely used in the fields of food and medicine.

The high output, low cost, short production time, and high production efficiency (Zhao *et al.* 2021) have made submerged fermentation (SmF) of edible and medicinal fungi become more effective in producing bioactive substances (Wu 2016). During SmF, the morphological architecture of mycelium is regarded as a key parameter. Two fundamental modes of hyphal branching exist: apical and lateral branching. Apical branching occurs directly at the growing hyphal tip with apical dominance to inhibit the formation of new branches in its vicinity (Semighini and Harris 2008; Harris 2019). The loss of apical dominance often leads to disorganized hyphal growth and consequently affects normal

colony expansion (Harris 2008). In contrast, lateral branching initiates from subapical regions and is typically separated from the primary hyphal tip by one or more septa (Harris 2008). Hyphae then form a mycelium through repeated branching. Mycelia exhibit a wide diversity of morphologies. The morphology can be one of three morphological forms, namely dispersed mycelia, clumped aggregates, or mycelial pellets (including rough and smooth pellets) (Kossen *et al*. 2000; Papagianni *et al*. 2004; Cairns *et al*. 2019b). Fungal morphology can be strongly and intricately determined by environmental conditions and inherent molecular or genetic biology. Apparently, pellets show slow growth and metabolism due to poor oxygen diffusion in the interior regions of large particles. In contrast, dispersed mycelium grows rapidly and has no limitations in nutrient transport (Li *et al*. 2026). Reports have shown that mycelium morphology in SmF was closely related to the synthesis of metabolites, and the optimal fermentation process often corresponded to the specific mycelial morphology (Veiter *et al.* 2018; Cairns *et al.* 2019a). For example, highly branched dispersed mycelium facilitates the production of protein-based products (Fitz *et al*. 2019), while organic acids, antibiotics prefer highly-branched pellets (Metwally 1998; Driouch *et al.* 2010; Lu *et al.* 2015; Yin *et al*. 2017). The flocculent mycelium is conducive to amylase and glycosylase production (Lu *et al*. 2015). The utilization of globular mycelium to keep low viscosity in fermentation broth for increasing the production of *L*-lactic acid is also a typical case (Zhao *et al.* 2013). The production of *Monascus* pigments by flocculent mycelium of *Monascus ruber* M7 was found to be the highest, which was 2.0 to 2.1-folds as high as that of spherical pellets, and 3.3 to 3.6-folds as high as that of clump aggregations (Yao *et al.* 2022). Tepwong *et al.* (2012) showed that the globular mycelium of *Lentinus edodes* was easier to synthesize ergothioneine compared with dispersed or clumped mycelium. Understanding and controlling mycelial morphology is of pivotal importance for fermentation process optimization to achieve efficient industrial production.

Polysaccharides of edible and medicinal fungi have diverse therapeutic effects in improving immunoregulation (Liao *et al.* 2015; Li *et al.* 2016; Gu *et al.* 2020), antitumor (Zhou and Chen 2011; Hyder and Dutta 2021), losing weight (Chang *et al.* 2017; Nakahara *et al.* 2019), and antiaging (Zhou and Chen 2011; Shi *et al.* 2017; Hyder and Dutta 2021) with little toxic side effects. They are international known as "biological response regulators". Due to their strong radicals scavenging capacity, edible and medicinal fungi polysaccharides have strong antioxidant effect (Hua *et al.* 2012; Wang *et al.* 2012). Lentinan can effectively alleviate mouse aging through scavenging free radicals, decreasing lipid peroxide and malonaldehyde (MDA) level, and increasing superoxide dismutase (SOD) activity (Zhang *et al.* 2010). Polysaccharides from *Morchella esculenta* fruit body can scavenge hydroxyl (OH·), DPPH and superoxide radicals, decrease reactive oxygen species, nitrogen oxygen and cell death, which could protect *Danio rerio* from oxidative damage (Cai *et al.* 2018). The research and exploitation of edible and medicinal fungi polysaccharide have attracted extensive attention in medicine, health products, and cosmetic industry.

Pleurotus ostreatus (Jacq.) P. Kumm., which belongs to the hierarchical classification of oyster mushrooms (Basidiomycota, Hymenomycetes, Agaricales, Pleurotaceae), is one of the most widely cultivated edible and medicinal fungi. In addition to immune-enhancing and antitumor activities, its polysaccharides also have strong antioxidant activity. Crude polysaccharides of *P. ostreatus* showed significant antioxidant activity *in vitro*. They can protect PC12 cells induced by H_2O_2 by inhibiting the contents of lactate dehydrogenase and MDA, increasing the levels of SOD and glutathione (GSH),

and the antioxidant protective effect was through the phosphatidylinositol 3 kinase (PI3K)/protein kinase B (Akt) signaling pathway and regulation of apoptosis-related pathway proteins (Wang *et al.* 2023). A water-soluble polysaccharide from *P. ostreatus* can improve GSH level and SOD activity of gastric ulcer mouse induced by acetic acid to ameliorate oxidative damage and enhance the gastric protective effect (Yang *et al.* 2012). As one natural antioxidant, application of *P. ostreatus* polysaccharides in the food and medicine fields has wide prospects, and production of polysaccharides raw material through SmF has industrial value. However, there have been no reports studying the relationship between mycelial morphology and the polysaccharides yield. During the fermentation process of *P. ostreatus*, two forms of mycelial morphology including globular or clumped mycelium appeared in different fermentation batches. In the present study, the goal was to comparatively study the difference of 1) mycelia biomass yield, 2) water-soluble crude endo-polysaccharides (*Po*-IPS) or exopolysaccharides (*Po*-EPS) production, 3) antioxidant activity of crude *Po*-IPS, and 4) characterization of the fermentation of pellet mycelium, for exploring the relationship between mycelial morphology of *P. ostreatus* and polysaccharides metabolism. The study was intended to provide data to demonstrate that mycelial morphology can affect the antioxidant activity of polysaccharides, and practical guidance for industrial production.

EXPERIMENTAL

Preparation of *Pleurotus ostreatus*

Pleurotus ostreatus (No. CICC50138) was purchased from China Center of Industrial Culture Collection. A piece of mycelia was inoculated onto potato dextrose agar (PDA, 200 g potato in boiling water for 30 min and filtrated through 4 layers of gauze to obtain extracting solution, glucose 20 g, KH_2PO_4 3 g, $MgSO_4 \cdot 7H_2O$ 1.5 g, agar powder 15 g in total 1 L water, and sterilized at 115 ℃ for 30 min). It took 5 to 7 days at 26 ℃ for the mycelium growth to fill the plate.

The mycelia that had passed through successive generations were scraped from PDA into 100 mL of potato dextrose broth (PDB) in a 250 mL conical flask, and incubated with shaking at 26 ℃, 160 rpm, for 7 d to prepare the primary inoculum. The initial pH was 5.32.

The detailed fermentation conditions were previously described (Sadiq *et al.* 2019). Briefly, 15% primary inoculum was inoculated into 400 mL sterile PDB in 1 L conical flask with sealing film, and three repeats were set. The fermentation was incubated at the same condition for 10 d. Every two days, 50 mL of fermentation solution was collected under sterile environment, pH was measured, and centrifuged at 4 ℃, 10000 rpm for 15 min. The fermentation broth was transferred to another new tube. Both fermentation broth and mycelia were stored at -80 ℃, then mycelia were freeze-dried and weighed. All samples were obtained from different fermentation batches.

ITS Amplification of *Pleurotus ostreatus*

DNA from mycelia on PDA, pellets, or clumped mycelium were isolated with an Ezup Column Fungi Genomic DNA Purification Kit (Sangon Biotech, Shanghai). The amplification of ITS fragment was run in a 20 μL reaction volume, containing 12.5 μL EasyTaq mix, ITS1/ITS4 primer (White *et al.* 1990) 1 μL, DNA 1 μL, and 9.5 μL of PCR-grade water. The profile was 95 ℃ 10 min; 95 ℃ 45 s, 54 ℃ 30 s, 72 ℃ 30 s, 35 cycles;

72 ℃ 10 min. The gel purified PCR product was linked into pMD-19T vectors, and linkage products were transformed into *Escherichia coli* DH5α for sequencing.

Crude Polysaccharides Extraction

The freeze-dried mycelia were crushed, dispersed in deionized water (1:20), and heated in water bath at 90 ℃ for 2 h. The extraction mixture was centrifuged at 12000 rpm for 20 min, and the supernatant was transferred to a new tube. The above steps were repeated two times with the precipitates again, and supernatants were collected together. Then, 3-folds volume of ethanol was added into supernatants. The mixture was stored at 4 ℃ for 24 h and then centrifuged at 12000 rpm for 30 min to harvest crude *Po*-IPS. The residue ethanol was volatilized in a drying oven at 40 ℃. The precipitates were dissolved in deionized water and freeze-dried. To obtain crude *Po*-EPS, 3-folds volume of ethanol was added into 20 mL fermentation broth. The other steps were the same.

Purification of Crude Polysaccharides

The Savage method (Hao *et al.* 2011) was applied to remove proteins from crude polysaccharides. Briefly, 3-folds volume of chloroform-butanol (v:v=4:1) were added into the crude polysaccharide solution, magnetic stirred for 2 h. The mixture was centrifuged at 5000 rpm for 10 min, and the upper water phase was taken out to repeat the above extraction process for 3 to 5 times until there were no white precipitates between the aqueous phase and the organic phase. Next, the decolorization of crude polysaccharides was carried out (Lin *et al.* 2016). The pH of crude polysaccharide solution after protein removal was adjusted to 8 with ammonia at 60 ℃ in a water bath, and 20% H_2O_2 was added and incubated at 45 ℃ for 3 h. The pH was adjusted to 7 again, and the mixture was centrifuged at 3000 rpm for 15 min to collect the supernatant. The supernatant was transferred into dialysis bag of 3.5 KDa for 72 h at 4 ℃, and deionized water was renewed every 6 h. After dialysis, the purified crude polysaccharides were freeze-dried.

Antioxidant Activity of Crude Polysaccharides

The freeze-dried crude polysaccharides were dissolved in deionized water at 60 ℃. The concentration was measured with phenol-sulfuric acid method, and the crude polysaccharide solution was adjusted to gradient concentration. Then the total antioxidant capacity (T-AOC), and the scavenging rate on OH· or DPPH radicals were detected according to the manufacturers' protocol (Solarbio, Beijing) through microspectrophotometry in a microplate reader.

Activity Detection of Various Decomposition Enzymes

The stored fermentation broth of pellet mycelium was thawed and centrifuged at 4 ℃, 12000 rpm for 20 min. The supernatant was prepared to detect various decomposition enzymes activity, including laccase (Lac), lignin peroxidase (LiP), manganese peroxidase (MnP), β-glucosaccharase activity (β-GC), peroxidase, and acid/neutral/alkaline proteinase. All the detections were performed according to manufacturers' protocols (Solarbio, Beijing).

Data Analysis

All data were given as mean ± SD (n = 3). The statistical analysis was performed with one way ANOVA by IBM SPSS Statistics 21. $P < 0.05$ or $P < 0.01$ was considered significant or highly significant, respectively.

RESULTS

The Morphology of Mycelium and Internal Transcribed Spacer (ITS) Identification of Globular or Clumped Mycelium

During the experiment process, two forms of mycelium under the same fermentation conditions in different batches appeared (Fig.1). One was globular mycelium (A), and the other was clumped mycelium (B). The morphology of globular mycelium can change during the whole fermentation process. The initial mycelium pellet was small (5.45 ± 3.30 mm) and rough with numerous radiating hyphae on their surface (2 to 3 d); then the pellet grew, hyphae on the surface broke off, and the texture of mycelium pellet became relatively hard (4 to 5 d). Subsequently, the mycelium pellet continued to grow (10.38 ± 7.65mm), the surface became very smooth, and the texture became brittle (6 d ~). The clumped mycelium kept a stable status, looking like soft down feather during the whole fermentation process (Fig. 1-B). To confirm that both forms of mycelium were *P. ostreatus*, ITS identification was performed (Fig. 1). Compared to the ITS amplification with DNA from mycelia on PDA as template, the same size bands were amplified by DNA from globular or clumped mycelium. Sequence alignments showed that the three sequences were the same; homologs analysis in the GenBank NCBI indicated they shared 100% identity with ITS of numerous *P. ostreatus* strains or isolates, which indicated the strain with two forms of mycelium are indeed *P. ostreatus*.

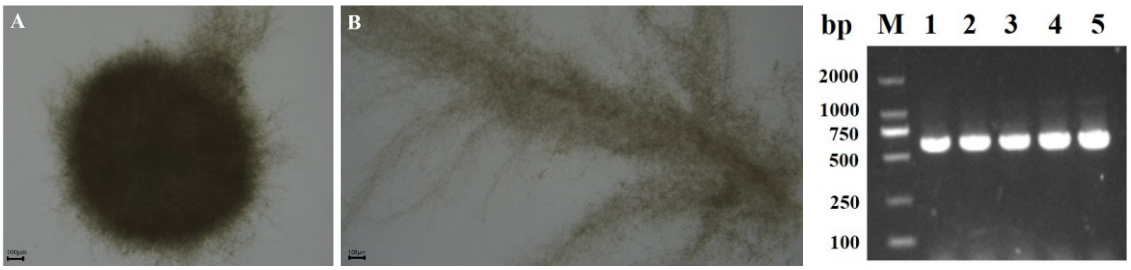


Fig. 1. Images of two forms of mycelium and agarose gel electrophoresis of ITS amplification. A: globular mycelium, B, clumped mycelium. Lane 1: ITS amplification from DNA of mycelia on PDA, lane 2-3: ITS amplification from globular mycelium DNA, lane 4-5: ITS amplification from clumped mycelium DNA. M: DNA marker

The Mycelia Biomass Yield in SmF of Globular or Clumped Mycelium

A 10-days SmF of *P. ostreatus* with two forms mycelium in different fermentation batches at the same conditions were carried out, respectively. The plot of mycelia biomass yield *vs.* time is shown in Fig. 2. Overall, the mycelial biomass increased gradually with fermentation going on, and then it began to decline. In the groups of globular mycelium with significantly different particle size (10.38 ± 7.65 mm), mycelia biomass reached the maximum value at 6 d of ~ 9.25 g/L, and kept ~9.19 g/L at 8 d (Fig. 2A). In the groups of globular mycelium with relatively uniform particle size (4.08 ± 1.28 mm), mycelia biomass reached the maximum production at 8 d of ~11.10 g/L, and it increased to >10 g/L at 6 d (Fig. 2B). Hence, the fermentation with relatively uniform and small size of globular mycelium had the highest mycelium yield.

Next, the same 15% inoculum volume with clumped mycelium was carried out (Fig. 3C). Data representing mycelia biomass showed no difference during the fermentation process. In the dynamic curve, the maximum yield emerged at 2 d of ~ 9.81 g/L. This may have been caused by the high inoculum volume. Therefore, 7.5% inoculum

volume with clumped mycelium was also carried out (Fig. 2D). The curve showed the mycelia biomass reached the peak value at 6 d of 10.73 g/L, but it achieved a production of 10.27 g/L at 4 d. The result indicated that the inoculum volume could affect the fermentation process of clumped mycelium, because higher inoculum volume itself brought more mycelia.

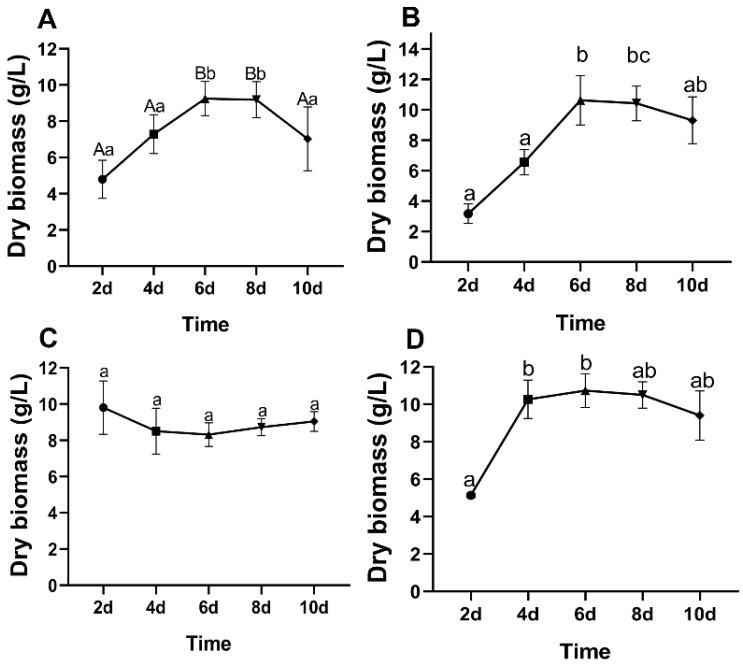


Fig. 2. Dynamic change of mycelia biomass in SmF of globular or clumped mycelium. The capital letters indicate highly significant difference (P<0.01), the small letters indicate significant difference (P<0.05), the same letters indicate no difference (P>0.05). A: mycelia biomass of globular mycelium with significantly different particle size, B: mycelia biomass of globular mycelium with relatively uniform particle size, C: mycelia biomass of clumped mycelium with 15% inoculum volume, D: mycelia biomass of clumped mycelium with 7.5% inoculum volume

Crude *Po*-IPS or *Po*-EPS in SmF of Globular or Clumped Mycelium

The production of water-soluble crude *Po*-IPS or *Po*-EPS in SmF is shown in Fig. 3. Their production presented a similar change trend in the same fermentation system of globular or clumped mycelium. In the groups of globular mycelium with relatively small and uniform particle size, *Po*-IPS increased rapidly, reached the maximum production of ~ 2.19 mg glucose equivalent /L at 6 d, then declined (Fig. 3B). In the groups of globular mycelium with significantly different particle size, *Po*-IPS production presented a similar change tendency, and obtained the peak yield of ~ 0.78 mg/ glucose equivalent L at 6 d, but there was no significant difference during the whole fermentation process (Fig. 3A). As to fermentation of clumped mycelium with 15% inoculum volume, *Po*-IPS production achieved a maximum value at 2 d of 1.47 mg glucose equivalent /L (Fig. 3C).

As to *Po*-EPS, its yield also reached a peak point of 3.66 mg glucose equivalent /L at 6 d, and there were no differences during the fermentation process in the groups of globular mycelium with significantly different pellet diameter (Fig. 3D). Meanwhile, *Po*-EPS production showed a high yield at 2 d of 4.33 mg glucose equivalent /L (Fig. 3E) and 9.07 mg glucose equivalent /L (Fig. 3F) in the groups of globular mycelium with relatively small and uniform particle size and clumped mycelium, respectively.

In sum, globular mycelium with relatively small and uniform particle size could produce 2.8-folds higher *Po*-IPS than that with significantly different particle size, and 1.5-folds higher than clumped mycelium. But clumped mycelium produced 2.09-folds higher *Po*-EPS. Namely, small and uniform globular mycelium was more favorable to the accumulation of IPS, while clumped mycelium was more conducive to generation of EPS.

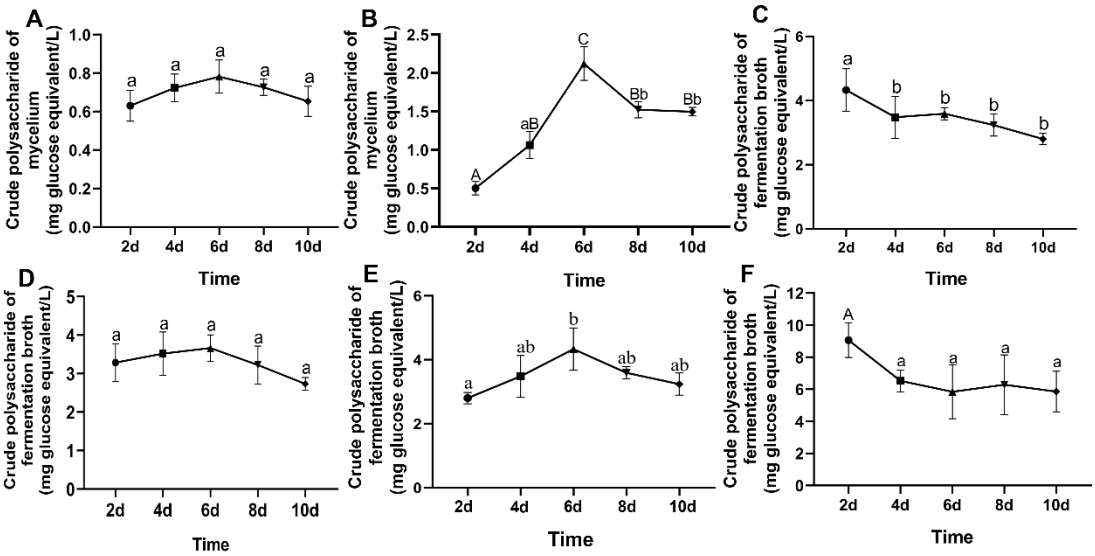


Fig. 3. Change of *Po*-IPS or *Po*-EPS production in SmF of globular or clumped mycelium. The capital letters indicate highly significant difference (P<0.01), the small letters indicate significant difference (P<0.05), and the same letter indicates no significant difference (P>0.05). A: *Po*-IPS production of globular mycelium with significantly different particle size, B: *Po*-IPS production of globular mycelium with relatively uniform particle size, C: *Po*-IPS production of clumped mycelium with 15% inoculum volume, D: *Po*-EPS production of globular mycelium with significantly different particle size, E: *Po*-EPS production of globular mycelium with relatively uniform particle size, F: *Po*-EPS production of clumped mycelium with 15% inoculum volume.

Antioxidant Activity of Crude *Po*-IPS from Two Forms of Mycelium

T-AOC of crude Po-IPS from two forms of mycelium

T-AOC of crude *Po*-IPS from globular or clumped mycelium is shown in Fig. 4. As the concentration of crude *Po*-IPS increased, T-AOC increased rapidly.

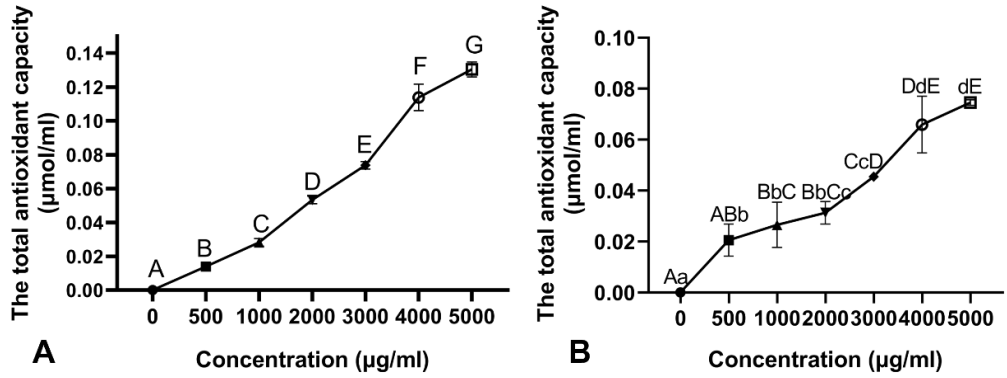


Fig. 4. T-AOC of crude *Po*-IPS from two forms of mycelium. The capital letters indicate highly significant difference (P<0.01), the small letters indicate significant difference (P<0.05), the same letters indicate no significant difference (P>0.05). A: T-AOC of crude *Po*-IPS from globular mycelium, B: T-AOC of crude *Po*-IPS from clumped mycelium.

The T-AOC of crude *Po*-IPS was concentration-dependent. Comparative analysis of A and B showed that T-AOC of crude *Po*-IPS from globular mycelium was higher than that from clumped mycelium when the concentration was greater than 1000 μg/mL.

The scavenging effect on OH· of crude Po-IPS from two forms of mycelium

Figure 5 shows the scavenging effect of crude *Po*-IPS on OH·. The scavenging percentage of crude *Po*-IPS from globular mycelium on OH· could reach more than 80%, and the scavenging effect did not follow a concentration-dependent manner but with a relatively constant level. In contrast, the scavenging efficiency of crude *Po*-IPS from clumped mycelium on OH· showed apparent concentration-dependent increasing trend, but the cleaning capacity was about 10-folds lower than that from globular mycelium.

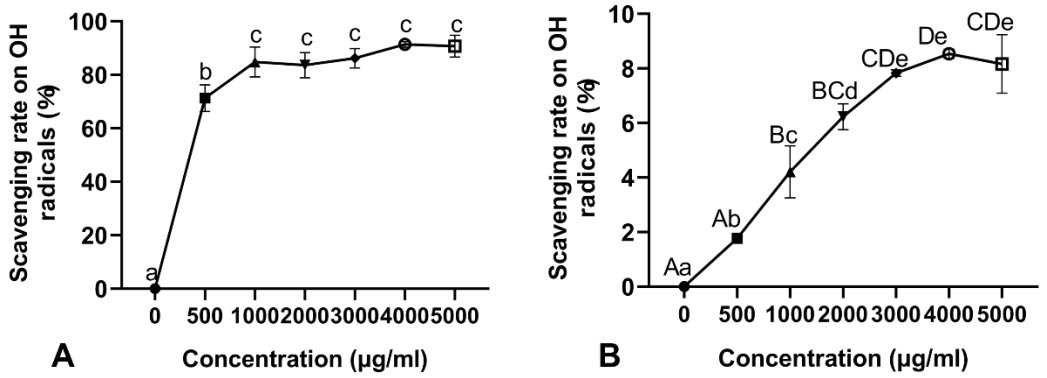


Fig. 5. The scavenging effect of crude *Po*-IPS from two forms of mycelium on OH·. The capital letters indicate highly significant difference (P<0.01), the small letters indicate significant difference (P<0.05), and the same letters indicate no significant difference (P>0.05). A: The scavenging percentage of crude *Po*-IPS from globular mycelium on OH·, B: The scavenging percentage of crude *Po*-IPS from clumped mycelium on OH·.

The scavenging effect on DPPH radical of crude Po-IPS from two forms of mycelium

The scavenging efficiency of crude *Po*-IPS on DPPH radical was assessed, and results are shown in Fig. 6.

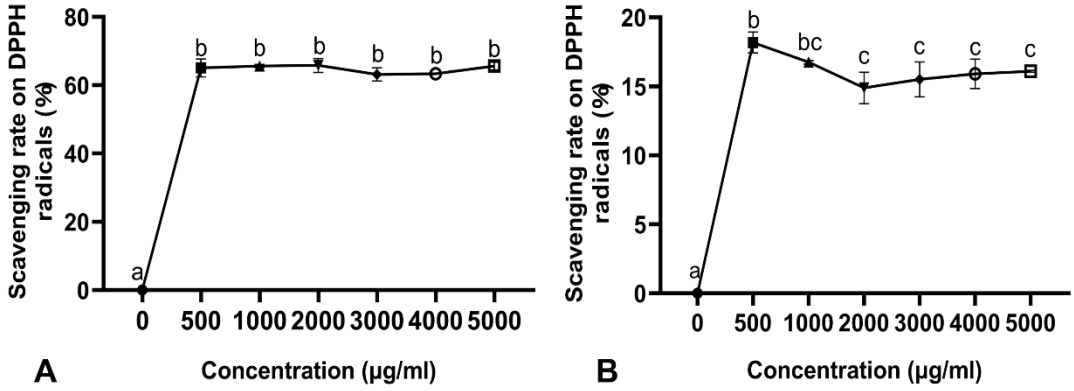


Fig. 6. The scavenging effect on DPPH radical of crude *Po*-IPS from two forms of mycelium. The small letters indicate significant difference (P<0.05), whereas the same letters indicate no significant difference (P>0.05). A: The scavenging % on DPPH radical of crude *Po*-IPS from globular mycelium, B: The scavenging % on DPPH radical of crude *Po*-IPS from clumped mycelium.

The scavenging effect on DPPH radical did not appear to depend on concentration. Compared to the scavenging percentage of crude *Po*-IPS from globular mycelium, the scavenging efficiency of crude *Po*-IPS from clumped mycelium was about 7-folds lower. As a result, it can be concluded that crude *Po*-IPS of globular mycelium had stronger DPPH radical scavenging activity. When considered together, *Po*-IPS from globular mycelium had stronger antioxidant activity. So the fermentation of globular mycelium was judged to be worth being characterized.

The Dynamic Change of pH in SmF of Globular Mycelium

The pH change during the whole SmF of globular mycelium in PDB was monitored, and the results are shown in Fig. 7. The initial pH was 5.32. The pH increased rapidly with fermentation progressed, reaching the maximum value of 6.40 at 8 d. The pH was already 6.38 at 6 d. The pH was in the acidic range during the whole fermentation process; pH 6.38 to 6.4 may be the optimal value for globular mycelium growing rapidly.

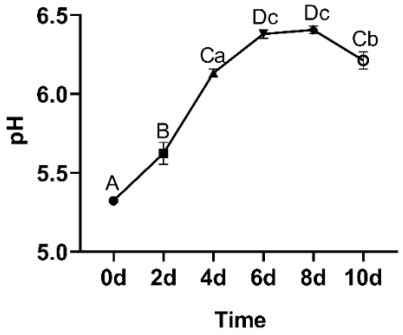


Fig. 7. The pH change in SmF of globular mycelium. The capital letters indicate highly significant difference (P<0.01), the small letters indicate significant difference (P<0.05), and the same letters indicate no significant difference (P>0.05).

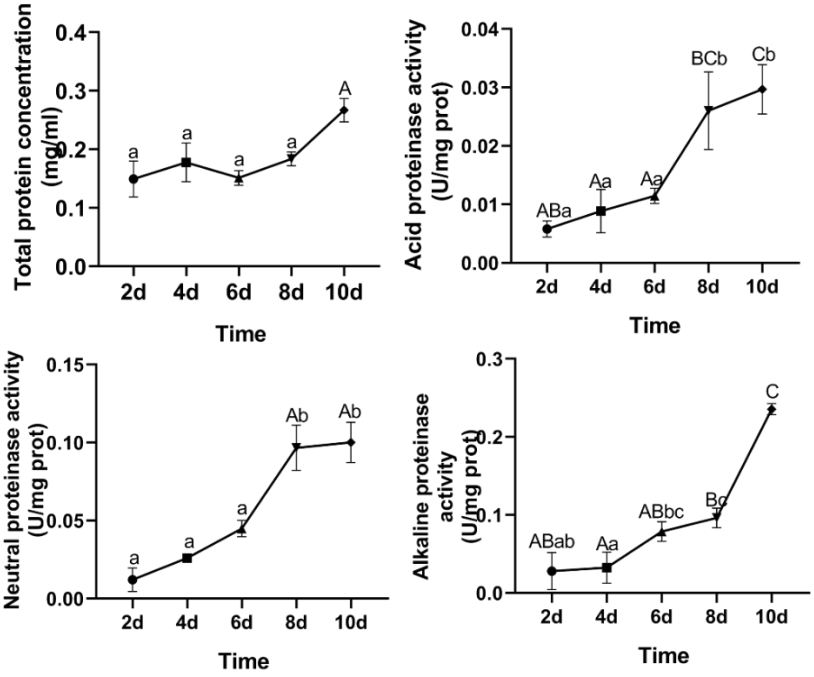


Fig. 8. The change of proteinases activity in fermentation broth of globular mycelium. The capital letters indicated highly significant difference (P<0.01), the small letters indicate significant difference (P<0.05), and the same letters indicate no significant difference (P>0.05).

Change of Proteinase Activity in Fermentation Broth of Globular Mycelium

Within the first 8 days, the total soluble protein level has no apparent change (P>0.05), and the significant increase just appeared at 10 d (Fig. 8-A). Acid, neutral, alkaline proteinase activity did not appear significant change within first 6 days, then they increased significantly at 8 d. Overall, the proteinase activity was relatively lower. Comparing these 3 proteinase activities, the strength of activity was as follows: alkaline proteinase>neutral proteinase>acid proteinase.

Change of Related Decomposition Enzymes Activity in Fermentation Broth of Globular Mycelium

Edible and medicinal fungi could secrete developed enzymes to degrade substrates for their own energy. Figure 9 shows the change of some decomposition enzymes activity during fermentation of globular mycelium in PDB.

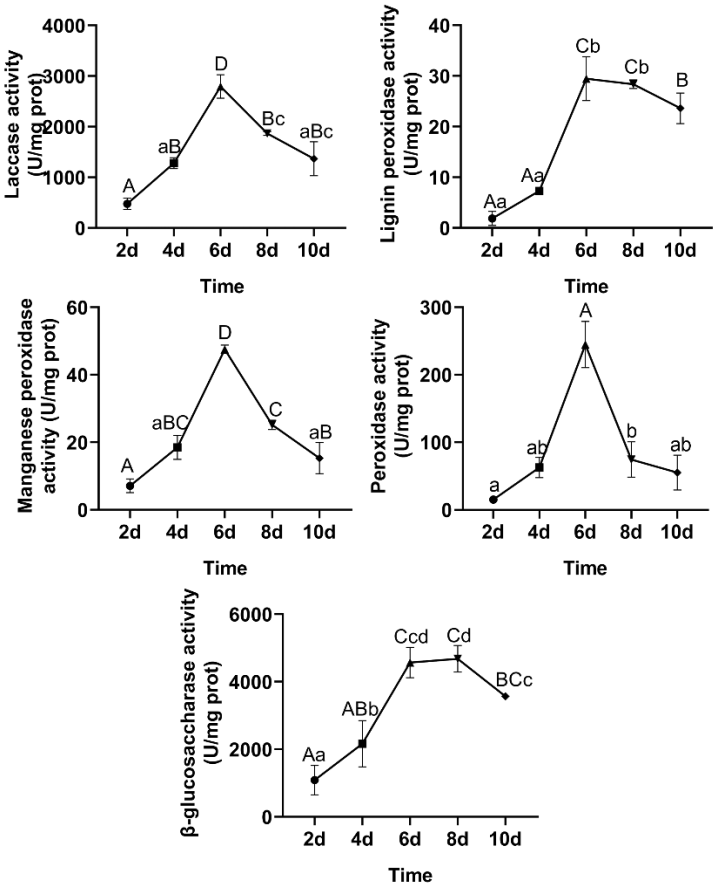


Fig. 9. The change of several decomposition enzymes activity in fermentation broth of globular mycelium. Capital letters indicate highly significant difference (P<0.01), the small letters indicate significant difference (P<0.05), and the same letters indicated no difference (P>0.05).

All detected enzymes activity showed a highly similar change pattern: they increased rapidly in the early fermentation stage, and reached the maximum activity at 6 d. Then the enzyme activity decreased rapidly as fermentation continued. Lac activity was the highest among all detected oxidases; it could reach as high as 2793.2 U/mg prot at 6 d, then peroxidase 245.0 U/mg prot, MnP 47.5 U/mg prot, and LiP 29.4 U/mg prot. In addition, β-GC belongs to carbohydrate active enzyme (CAZy). The maximum β-GC

activity was higher than Lac activity; it could go up to 4567.8 U/mg prot at 6 d, and continued a small increase to 4677.8 U/mg prot at 8 d.

Therefore, in the fermentation of globular mycelium, the dynamic change of decomposition enzymes activity presented a highly similar trend to mycelia biomass yield, polysaccharides production. The 6th day marked the peak of the fermentation process.

DISCUSSION

There have been few studies on the relationship between mycelium morphology and metabolites in edible and medicinal fungi, and they mainly have focused on mold (Papagianni 2004). Recent reports showed that mycelium morphology in SmF of edible and medicinal fungi was closely related to metabolites, this was true in *Aspergillus niger* (Wucherpfennig *et al.* 2011) and *Ganoderma lucidum* (Ding *et al.* 2012). Mycelium morphology is a pivotal indicator in SmF of filamentous fungi, and specific mycelium morphology generally corresponds to the biosynthesis of certain metabolite. The overall production of *P. ostreatus* fruiting body in China has reached the leading level of the world, and studies on SmF have been carried out. However, there has been no report focusing on its mycelium morphology. The exploration that mycelium morphology might affect functional activity of active metabolites has been even less in the study of filamentous fungi. In this study, two forms of mycelium appeared in the different fermentation batches at the same conditions. Such differentiation may be caused by the storage of solid-cultivated mycelium, or genetic instability of the strain. In SmF, mycelium is the most intuitive material. Mycelium biomass is one of the key indexes in SmF. The mycelia biomass of *P. ostreatus* in globular mycelium fermentation batches showed a trend of first increasing and then decreasing, which was consistent with the growth characteristic of most filamentous fungi. The maximum biomass reached ~ 9.25 g/L at 6 d. While the mycelium morphology was clumped, the maximum biomass yield of 9.81 g/L was obtained just at 2 d with 15% inoculum volume, and 10.73 g/L at 6 d with 7.5% inoculum volume. The clumped mycelium itself is relatively dense, so the larger primary inoculum (15% in the study) could bring more biomass. However, a report indicated that the mycelial biomass of *P. ostreatus* could reach 22.86 g/L at 4 d, but they did not describe the morphology of mycelium (Tun Ni Sa 2019). Compared to the compositions of the two media, it is apparent that the media in the report included added peptone, which might be the main reason for the high yield, because peptone is a commonly used and preferred nitrogen source in SmF of filamentous fungi. Peptone can also affect mycelium morphology (Wucherpfennig *et al.* 2011; Ding *et al.* 2012). As the peptone concentration increased, the mycelium pellet of *A. niger* (Wucherpfennig *et al.* 2011) became bigger, the dried biomass yield of *G. lucidum* increased to 5.41 g/L at 40 g/L peptone. Screening the optimal combination of medium components with lower cost shaping the specific morphology to obtain the maximum yield would be an important work needed to be carried out in future for industrial production.

In the study, the pattern of crude polysaccharides production extracted in hot water was consistent with mycelia biomass yield. Although there was no significant difference in the mycelial biomass yield between globular and clumped mycelium fermentation, water-soluble crude *Po*-IPS production from globular mycelium was 1.5-folds higher than that from clumped mycelium, while water-soluble crude *Po*-EPS production from clumped mycelium fermentation was 2.8-folds higher than that from globular mycelium. Hence, clumped mycelium of *P. ostreatus* may have morphology superiority to produce more

water-soluble EPS. Wagner *et al.* (2004) showed that the polysaccharides of *G. lucidum* went into the rapid production period when mycelium morphology transformed from globular to be clumped; the increase in the diameter of *G. lucidum* mycelium pellet was unfavorable for production of EPS. Mycelium pellet of *Poria cocos* with medium size and rough surface is more favorable of EPS accumulation (Papagianni 2004). The activity of lipase produced by *Rhizopus chinensis* is the lowest when the mycelium is dispersed, while its activity increases 6 folds at the form of clumpy mycelium (Teng *et al.* 2009). The mycelium morphology of *A. niger* transformed from globular to be clumped, glucosamine production decreased significantly (Papagianni and Mattey 2006). In SmF of *P. ferulae*, globular mycelium is more beneficial to the production of Lac than clumped mycelium, and Lac activity is 2.5-folds higher than that of dispersed mycelium (Chen *et al.* 2013). In brief, mycelial morphology plays a key role in regulating the synthesis of target products.

At the backdrop of an increasing global aging population, anti-aging has become a social issue of concern. The chemical synthesized antioxidants generally have side effect, the development of some natural antioxidants that could eliminate free radicals has been a hot topic in recent years. Edible and medicinal fungi polysaccharides are precisely such candidates. Polysaccharides extracted from *Lepista nuda* and *Oudemansiella radicata* using hot water showed the increase of scavenging activity to DPPH, $O^{2-}\cdot$ and OH· with increasing concentration (Wang *et al.* 2018; Shu *et al.* 2019). Reports also indicated *Pleurotus* polysaccharides (*P. cornucopiae*, *P. eryngii*, *P. nebrodensis*) could scavenge OH·, $O^{2-}\cdot$ and DPPH radicals, and the scavenging percentage was about 20% (Liu *et al.* 2010). The water-soluble polysaccharide with molecular weight of 2.3×10^4 Da from *P. ostreatus* (POPw) could significantly inhibit the mucosal damage of gastric ulcers rat induced by acetic acid, and improve GSH and SOD activity, the result demonstrated that the gastroprotective effect of POPw on rat ulcer model can be attributed to its amelioration of oxidative damage (Yang *et al.* 2012). Similarly, the scavenging activity of free radicals by *Po*-IPS can be more than 60%, which provided further support for its antioxidant potential.

The increase of polysaccharides production by control mycelial morphology is important for practical applications. Maintaining or even improvement of its functional activity might be even more important. However, most of the present studies focused on the upstream work to increase the target metabolites production without taking activity into account. In the present study, the relationship between mycelial morphology and antioxidant activity of crude polysaccharides was first explored. The scavenging rate on OH· or DPPH radicals and T-AOC of crude *Po*-IPS from globular mycelium are considerable higher than that from clumped mycelium. As a consequence, crude *Po*-IPS obtained from pellet mycelium possessed stronger antioxidant activity. The morphology of filamentous fungi is complex, and the change of morphology is closely related to cell metabolism and products synthesis (Cui *et al.* 2016). The change of mycelial morphology impacts the supply of dissolved oxygen, heat and mass transfer, and rheological properties of fermentation broth (Wucherpfennig *et al.* 2010). Clumped mycelium increases the viscosity of fermentation broth, hindering the dissolve, transfer and uniform distribution of substrate and oxygen, which will affect mycelium growth and metabolites synthesis (Veiter *et al*. 2018). Globular mycelium has better substrate and heat transfer effect (Calvo *et al.* 2002). In particular, a quantity of EPS can be produced in SmF of most edible fungi, which can also affect the rheological properties of fermentation broth in some degree. Moreover, the change of mycelial morphology might lead to change in metabolic pathways, the generation of harmful metabolites, and even the cellular autolysis, which will further accelerate the change of morphology. Therefore, the mutual influence between mycelial

morphology and culturation environment can cause change of anabolism, which will result in subtle changes of the structure or modification of some metabolites, including polysaccharides. The activity is closely related to their structures. The next work will focus on the comparative analysis of *Po*-IPS structure, screening out some certain structures or modifications which might be applied for artificial modification to improve antioxidant activity of polysaccharides. In addition, exploring some means which could be applied to control the mycelial morphology is of important practical significance. Hence, that specific mycelial morphology always corresponds to certain target metabolite is almost accepted, and the theory that mycelial morphology also affects the activity of targeted product will be revealed in future.

The pH is a pivotal factor influencing SmF. Inappropriate pH during the fermentation will hinder the growth of mycelium, inhibit some certain enzymes activity, and affect the mycelium morphology (Li *et al.* 2013). Edible and medicinal fungi depend on their secreted enzyme system in different growth stages to utilize external nutrients for energy, and these enzymes have their own optimal pH range. The decomposition enzymes system synthesized by white rot fungi *P. ostreatus* mainly includes Lac, LiP, MnP, and CAZy to decompose lignin into CO_2 and corresponding monosaccharides for their growth (Levasseur *et al.* 2013; An *et al.* 2016b). In the study, the pH range of 6.38 to 6.40 was found to be the optimal range for the highest enzyme activity activation, which will promote mycelium growth and polysaccharide accumulation of globular mycelium fermentation. While lac was the main one, Lip, MnP, and other oxidases helped to decompose the starch in medium effectively to be oligosaccharides. β-GC then decomposed oligosaccharides into glucose for final utilization by mycelia. The activity of the degrading enzymes is closely related to the degradation rate of substrates; it can reflect the biological conversion rate and growth rate of mycelium (Yang and Wyman 2006; Qin and Shuai 2008). *Pleurotus* strains are easier to use starch substance as carbon source, because their degrading capacity to lignocellulose is low, and they prefer to degrade starch substance for their carbon source and energy requirement in the initial stage of culture (Xu *et al.* 2014). Different cultivation substrates affect the activity of degrading enzymes, but the overall change trend remained consistent (Liu *et al.* 2006). In the late stage of fermentation, the decline of pH is caused by organic acid production. Then the decrease of enzyme activity, the reduction of nutrients, the accumulation of metabolic wastes, and the deterioration of rheological property can cause the permeability of cell membrane, or even cellular autolysis (Yang and Wyman 2006). Simultaneously, due to the continual consumption and reduction of nitrogen or carbon source in the medium, mycelia begin to secret proteinase to degrade protein (mainly enzymes) for nitrogen utilization, or even they might decompose some their own tissue structures for themselves energy requirement. All these changes of metabolism finally showed on the growth of mycelium and metabolites synthesis (Leong *et al.* 2021).

CONCLUSIONS

1. A period of 6 days reached the end of fermentation of *P. ostreatus* in SmF, but inoculum volume could affect the time.

2. Mycelial biomass did not show a significant difference between globular and clumped mycelium. Globular mycelium could produce more IPS, while clumped mycelium produced more EPS.

3. The antioxidant activity of IPS from globular mycelium was considerable higher than that from clumped mycelium.

4. During the fermentation of globular mycelium, pH 6.4 is the optimal for various enzymes activity activation to decompose the starch for their own nutrients and energy utilization.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Use of Generative AI

The authors declare that they did not use AI in the paper.

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APPENDIX

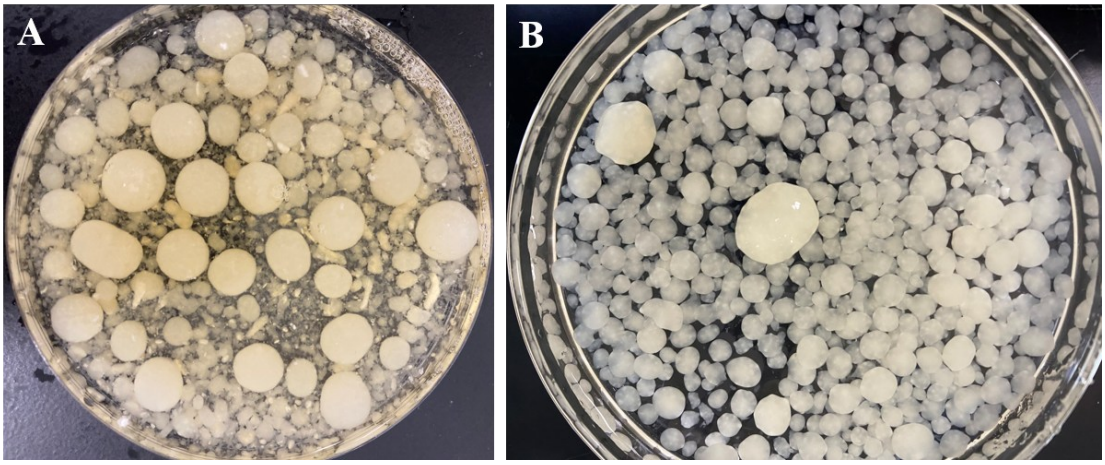


Fig. S1. The different size of pellet of globular mycelium