



**Full Length Article**

## Cultivation of the Wild *Ganoderma gibbosum* Mycelia via Submerged Liquid Fermentation for Inter/Extra-Cellular Polysaccharides

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### Abstract

*Ganoderma* are well-known for their healthy functions in China. In this work, a wild species of Huoshan, *Ganoderma gibbosum*, was successfully cultured from fruiting body. On solid culture media, mycelia grew and covered almost whole plates at 7<sup>th</sup> day; while the maximal biomass of mycelia (3.9 g/L) was obtained at 11<sup>th</sup> day during liquid submerged fermentation process. Microscopic observation showed that vegetative hypha had several septa and aerial hyphae with spores were formed at about a week's incubation. SEM studies showed that mycelia grew with a pellet morphology that changed with the incubation time. The contents of the intercellular polysaccharides reached a maximum value of 2.97% at 15<sup>th</sup> day; while the extracellular polysaccharide contents were up to the biggest value of 34.12% at the same period during the liquid submerged fermentation. It is important to explore, cultivate and utilize the wild resources of *Ganodermataceae*, especially for culture collection of the distinctive germplasm resources. © 2018 Friends Science Publishers

**Keywords:** *Ganoderma gibbosum*; Mycelia cultivation; Liquid submerged fermentation; *Ganoderma* polysaccharides

### Introduction

*Ganoderma*, known as the “celestial beings grass”, are the ancient and immortal Chinese medicinal herbs. *Ganoderma* are widely distributed in Asian countries, especially in China, Korean Peninsula and Japan. As one of the frequently used fungi in Chinese medicine and nutrition, *G. lucidum* commonly famous as Chinese Lingzhi, is the most reported fungus in recent years (Bishop *et al.*, 2015). Variety of bioactivity substances like *Ganoderma* polysaccharides (Askin *et al.*, 2010; Ferreira *et al.*, 2015), ganodenic acid, *Ganoderma* triterpenoids (Wu *et al.*, 2013) can be produced by *G. lucidum*, making it a “factory” of bioactivator producer (Shiao, 2003). As the functional components, *Ganoderma* polysaccharides have favourable bioactivities e.g., attenuation of microglia-mediated neuroinflammation (Cai *et al.*, 2017), promotion of cognitive function and neural progenitor proliferation (Huang *et al.*, 2017), restoration of the tumor-suppressor function to mutant p53 (Jiang *et al.*, 2017), reducing obesity in mice (Chang *et al.*, 2015), anti-inflammation, anti-oxidation and reducing hepatic lipid effects (Chung *et al.*, 2017) and stimulation for the macrophage proliferation (Shi *et al.*, 2013). Another common species of *G. atrum* has functions of amelioration of anoxia/reoxygenation-mediated oxidative stress and apoptosis (Zhang *et al.*, 2017), induced activation and maturation of murine myeloid-derived

dendritic cells (Wang *et al.*, 2017), served as mannose receptor mediation of the immune response in macrophages (Li *et al.*, 2017), inducing growth inhibition and cell death in breast cancer cells (Xu *et al.*, 2016), antioxidant action and short-chain fatty acids excretion (Zhu *et al.*, 2016) and neuroprotective effects (Qiu *et al.*, 2016). However, most of these bioactive constituents are extracted from the fruiting bodies. With the growing of population and expansion of human activities, the forest resources are dwindling year by year. In this situation, the wild resources of *Ganodermataceae* become less and less. Hence, protection of the distinctive germplasm resources and exploration of the novel cultivation method is urgently needed at this moment.

Throughout the ages, artificial cultivation and mycelia fermentation of the distinctive germplasm resources of *Ganoderma* species are good strategies for production of bioactivity substances, such as *Ganoderma* polysaccharides, ganodenic acid. As the major bioactive components, *Ganoderma* polysaccharides have attracted tremendous attentions due to their unique healthy functions, such as the immunomodulatory activity of *G. atrum* polysaccharide (Xiang *et al.*, 2017), the neuroprotective effects (Sun *et al.*, 2017), antidiabetic activity (Xiao *et al.*, 2017) and anti-oxidation and hepatoprotective efficacy (Chiu *et al.*, 2017) of *G. lucidum* polysaccharide, the antitumor activity of *G. applanatum* polysaccharide (Sun *et al.*, 2015), and so

on. In recent years, with the increasing of requirement artificial cultivation of *Ganoderma* enlarges year by year, however, due to the limitation of culture conditions, e.g., long growth period, multitudinous external environmental influencing factors, the yield could not meet the demands in the market place. Therefore, the liquid submerged fermentation technique could be a good strategy to obtain the *Ganoderma* polysaccharides in large-scale.

As the rare resources, Huoshan *Ganoderma* sp. are well-known for their healthy functions, which are also the products of the National Protection of Geographical Indications of China. In the present work, a fruiting body of the wild *G. gibbosum* was collected from Huoshan County. Very few papers have been reported on this species for the moment. Pu *et al.* once isolated eight new kinds of gibbous acids A-H (1–8) from the fruiting body of *G. gibbosum*, which were collected from Ciba County, Yunnan Province (Pu *et al.*, 2017). As the scarce and distinctive wild germplasm resource, we want to culture and preserve this kind of species via mycelia cultivation, and further to obtain the bioactive substances like *Ganoderma* polysaccharides through the liquid submerged fermentation technique in this work. The mycelia morphology, growth characteristics and polysaccharides contents were investigated.

## Materials and Methods

The fruiting body of wild *G. gibbosum* was a kind gift of Mr. Yin-He Li, which was collected from Huoshan county, Lu'an city, Anhui Province, P. R. China. The specimen was identified by Prof. Ye-Shou Shen, who is the president of Anhui Edible Fungi Technique Association. The solid culture medium (SCM) contained: potato, 200 g/L; glucose, 20 g/L;  $MgSO_4 \cdot 7H_2O$ , 1.5 g/L;  $KH_2PO_4$ , 3 g/L; Vitamin B<sub>1</sub> 10 mg/L; agar, 20 g/L. And the liquid culture medium (LCM) contained: sucrose, 20 g/L; yeast extract, 1 g/L;  $KH_2PO_4$ , 3 g/L;  $MgSO_4 \cdot 7H_2O$ , 1.5g/L; Vitamin B<sub>1</sub> 10 mg/L. Chemicals with analytical grade were used in all experiments. The procedure for maintenance and processing of *G. gibbosum* has been described as under:

**Activation and Purification of the Mycelia:** The mycelia of *G. gibbosum* were activated and then purified with SCM in plates. A small piece of the fruiting bodies of the wild *G. gibbosum* was cut off and scrubbed with 75% (v/v) ethanol, then incubated on the plate culture media at 28°C for five days. The obtained mycelial cultures were incubated on the fresh plate culture media under the same conditions. Repeat the procedures to purify the mycelial cultures for 3~5 times, and the purified cultures were preserved in test tubes at 4°C freezer and subcultured for relative experiments.

**Subculturing of the Purified Mycelia:** The purified mycelia were further inoculated in SCM plates under same conditions to observe the colony shape in the process of growth. At predetermined time intervals, the colonial shapes were photographed with a digital single lens reflex (DSLR) camera (Canon EOS 600 D, Canon Co.,

Japan). The mean diameter size of the colonies was determined with the DSLR images, which were analyzed through the analysis software (Nano Measurer, Department of Chemistry, Fudan University). Meanwhile, the mycelium morphology was observed by inverted fluorescence microscope (Olympus IX73, Olympus Optical Co., Ltd., Japan).

**Liquid Shake-flask Fermentation of the Mycelia:** The purified mycelia preserved were further amplified and cultured with SCM in test tubes at 28°C for five days. The obtained mycelia of *G. gibbosum* were washed with fresh LCM in the test tubes under sterile conditions. The washing solutions (2 mL) were incubated into shake-flasks contained 200 mL fresh LCM in each bottles. The inoculated solutions were fermented in shake-flasks at 28°C, 120 rpm for fifteen days. The mycelia morphology during growth was observed with the DSLR camera in a certain period of time.

**Morphological Characterization of the Mycelia:** To investigate the mycelia morphology variation during liquid fermentation process, the mycelia were observed with scanning electron microscope (SEM, S-4800, Hitachi, Japan). At predetermined time intervals, two to three mycelia pellets were sampled with pipettor gently and washed with distilled water for three times, the tip's end was cut with larger caliber. The samples obtained were freeze-dried (CHRiST Freeze Drier, ALPHA 1-4 LD plus, Martin Christ Gefriertrocknungsanlagen, Co., Ltd., Germany) for 12 h. The dried mycelia pellets were sputter-coated with gold before observation.

**Growth Curve Measurement of the Mycelia:** The growth curve of mycelia during liquid fermentation process was measured by dry weight method. According to the liquid fermentation process, 24 shake-flasks each contained 200 mL fresh LCM each were inoculated with the purified mycelia and incubated at 28°C, 120 rpm for fifteen days. At predetermined time intervals, three bottles were sampled randomly. The mycelia obtained in shake-flasks were collected via filter membrane suction method, and washed with distilled water for three times. The filters were preserved in 4°C freezer for further studies. After all of the samples were collected, the filters were dried at 80°C and weighed to a constant weight. All of the measurements were performed in triplicates.

**Extraction of Crude Polysaccharides:** The crude polysaccharides that produced by mycelia during liquid fermentation process were measured via water and ethyl alcohol method. The dried mycelia were collected according to the procedures that performed in growth curve measurement of the mycelia, and used to extract the intercellular polysaccharides. A suitable amount of the dried mycelia were ground sufficiently, added four times volume of deionized water, mixed well and put into water bath (90°C) for 120 min, stirred once per 20 min. The mixed solution was filtered by membrane suction method; the filter residue was re-extracted for two to three times under the same procedures as above mentioned. The

collected extraction solution was concentrated via rotary evaporation. Four times volume of absolute ethanol was added to the concentrated solution, stirred well and put into 4°C freezer for 12 h. The precipitates were collected through centrifugation (4000 rpm, 10 min), then dissolved the precipitates in distilled water to obtain the crude intercellular polysaccharides. The proteins in the crude polysaccharides solution were removed via Sevage method, the volume of crude polysaccharides solution to chloroform to butanol was 5:4:1, collected the supernatant after centrifuge, repeat the procedures for three to four times until no proteins were detected. Little molecular substances were removed via dialysis method, collected the purified crude polysaccharides solution and freeze-dried (CHRiST Freeze Drier, ALPHA 1-4 LD plus, Martin Christ Gefriertrocknungsanlagen, Co., Ltd., Germany) for 12 h to get the dried samples.

The crude intercellular polysaccharide's content in the dried samples was measured by phenol-sulfuric acid method, 6% (v/v) phenol, strong sulfuric and 100 µg/mL glucose standard solution were used. Phenol (1 mL, 6%, v/v) and strong sulfuric (5 mL) were added to the standard diluted glucose solutions, respectively. The mixed solutions were reacted for 30 min at room temperature, and then measured the absorbance at 490 nm. Draw the standard curve and acquire the regression equation to calculate the crude intercellular polysaccharide's contents.

Meanwhile, the culture media were also collected, filtrated and concentrated via rotary evaporation. The concentrated solution was freeze-dried (CHRiST Freeze Drier, ALPHA 1-4 LD plus, Martin Christ Gefriertrocknungsanlagen, Co., Ltd., Germany) for 12 h to get the dried samples. The same procedures were performed to extract the extracellular polysaccharides from the dried samples as mentioned above.

### Statistical Analysis

Date analyses were performed using the OriginLab software (OriginPro 9.0.0, OriginLab Co., USA). One-way analysis of variance followed by the Tukey's test was used to evaluate the statistical significance.  $P < 0.05$  was regarded to be statistically significant. Experimental results were expressed as mean  $\pm$  standard deviation (SD). All measurements were performed at least in triplicate.

## Results

### Solid Culture of the Fruiting Bodies of *G. gibbosum*

As a kind of wild *G. gibbosum* that picked from mountain forest (Fig. 1 on the top left), the germplasm resource preservation is a worthwhile work to be done. Our group has always worked to establish a culture collection holding room of distinctive edible fungi germplasm resources for Anhui Province. Some kinds of the precious rare edible fungi have been successfully cultured in our laboratory,

such as *Grifola frondosa* (Zhang *et al.*, 2016), *Sarcodon aspratus* (Chen *et al.*, 2013), *Morchella esculenta* (Hu *et al.*, 2013), and their cultivation, preservation and functions been well studied.

In the present work, mycelia of the fruiting body of *G. gibbosum* were successfully cultured with the solid media in 28°C incubator. The purified mycelia could be preserved in test tubes at 4°C freezer (Fig. 1 on the top right). Transfer generation experiments indicated that the characters of the purified mycelia were stable. Morphology observation showed each of the vegetative hypha had several septa (Fig. 2a), the aerial hyphae were formed after about a week's incubation, and the spores could be seen clearly (Fig. 2b). SEM images show the mycelia enwound together during the growth and proliferation (Fig. 2c and d). These physiological phenomena were accordance with the basic characteristics of the *G. gibbosum* growth (Chen *et al.*, 2010), similar results were also found in other species of *Ganoderma*, for instance, the Fed-Batch processing of *G. lucidum* reported by Fazenda *et al.* (2010), the mycelial growth of *G. applanatum* reported by Jo *et al.* (2009) and Kadowaki *et al.* (2011).

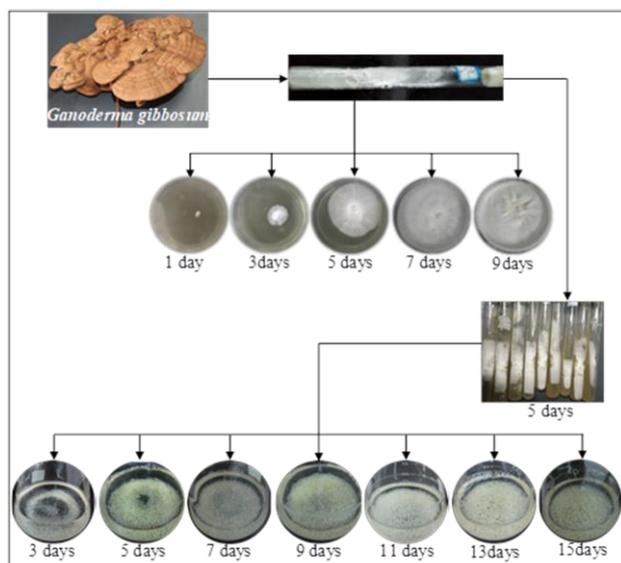
The purified mycelia colony on the SCM plates assumed the yellow white color with discoid shape (Fig. 1 on the second row). After a week's growth and proliferation, the growth of mycelia covered almost the whole plate, the diameter of mycelia colony presented an increasing trend with increasing of incubation time (Fig. 3), and it was  $95.15 \pm 0.25$  mm at the seventh day. As can be seen from Fig. 3, the mycelia were still in growth and not into stationary phase.

### Liquid Submerged Fermentation of the Mycelia

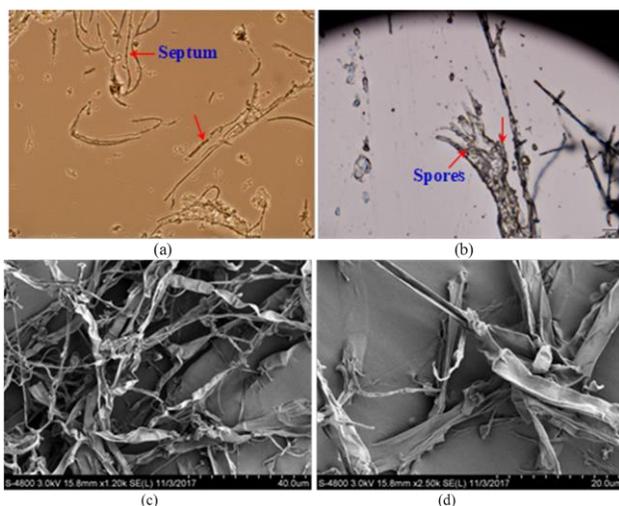
To enlarge cultivation of the purified mycelia and acquire the useful metabolic products, such as *Ganoderma* polysaccharides, the submerged fermentation technique was used. As can be seen from Fig. 1 (on the last row), the mycelia grew and proliferated well under the LCM conditions, it formed small balls after three days incubation, the pellets grew bigger with increasing of incubation time. During the liquid submerged fermentation, the growth of fungal hypha was inclined to aggregate together, which was a normal physiological phenomenon. Similar results were found in lots of research reports, especially in the species of *G. lucidum*, such as the pellets that formed in the liquid fermentation of *G. lucidum* (Liu *et al.*, 2016; Wan-Mohtar *et al.*, 2016; Ren *et al.*, 2017). However, it's rarely reported in other species of the genus of *Ganoderma* by now.

### Growth Characteristics of the Mycelia

Fig. 1 (on the second row) shows the mycelia covered almost the whole plate in about 7 day's growth and proliferation, how about later incubation? To investigate the growth characteristics of the mycelia in a whole cycle, the growth curve of the mycelia pellets of *G. gibbosum* during

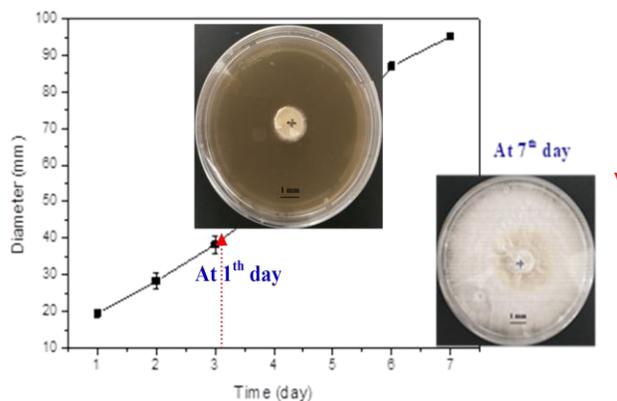


**Fig. 1:** Schematic illustration of cultivation of the mycelia from the fruiting body of *G. gibbosum* with the solid media and then fermented via the liquid submerged technique

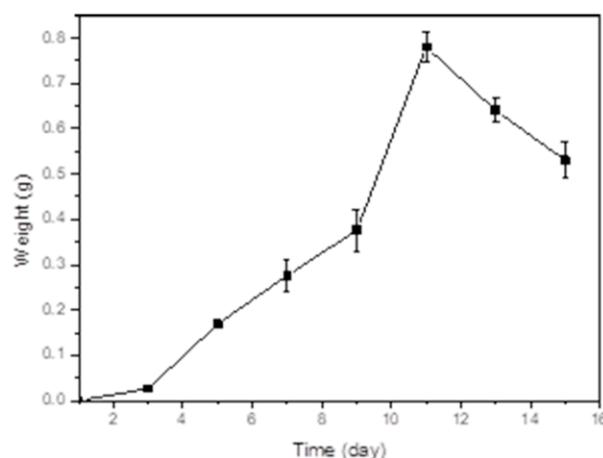


**Fig. 2:** Mycelia morphology observation of *G. gibbosum* in solid medium's cultivation. Optical micrographs of (a) the vegetative hyphae with septa and (b) the aerial hyphae with spores; SEM images of the mycelia during the growth and proliferation (c–d)

liquid submerged fermentation process was measured. As can be seen from Fig. 4, the growth of the mycelia was in late lag stage during the first three days after incubation, the mycelia were adapt to the novel survival conditions (LCM) at this period. After 3 days, the mycelia were in logarithmic phase till 11 days, the dried weight of the mycelia was up to  $0.78 \pm 0.03$  g per 200 mL culture



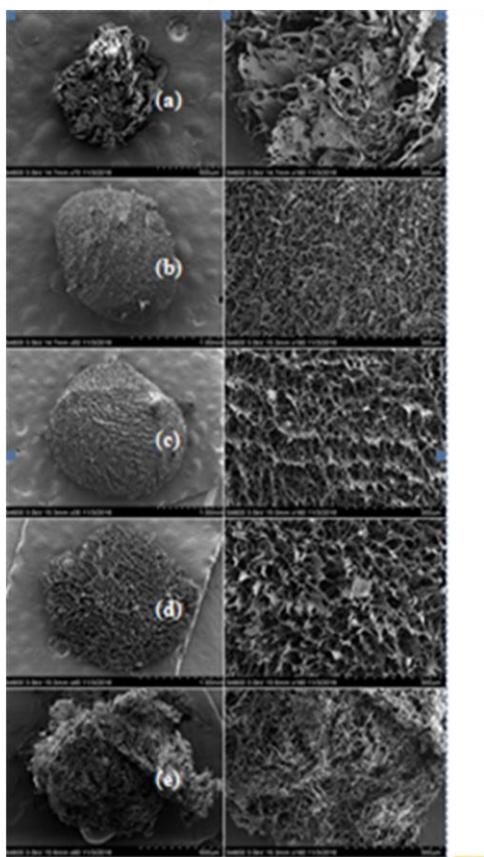
**Fig. 3:** Growth variation of the mycelia colony of *G. gibbosum* during the solid medium's cultivation. Each scattergram is expressed as mean  $\pm$  SD from  $n=3$



**Fig. 4:** The growth curve of the mycelia pellets of *G. gibbosum* during the liquid submerged fermentation process. Each scattergram is expressed as mean  $\pm$  SD from  $n=3$

medium. The mycelia were in the rapid growth and proliferation at this period (3–11 days), and then the mycelia growth was entered the decline phase.

During the liquid submerged fermentation process, the mycelia grew with a pellet morphology that changed with the incubation time. As showed in Fig. 1 (on the last row), with the fermentation time's increasing the total mycelia pellets were increasingly growing in number, which was ascribed to the 2<sup>nd</sup>-generation, 3<sup>rd</sup>-generation, etc. pellets release (Wagner *et al.*, 2004). Hereby, SEM was used to observe the morphology changing of the pellets growth. Fig. 5a shows the pellet that was formed after 1 day's incubation, the structure of the pellet was loose due to fewer mycelial proliferation and aggregation. With the increasing of the incubation time, the structure of the pellet became more and more regular and tight compared with the

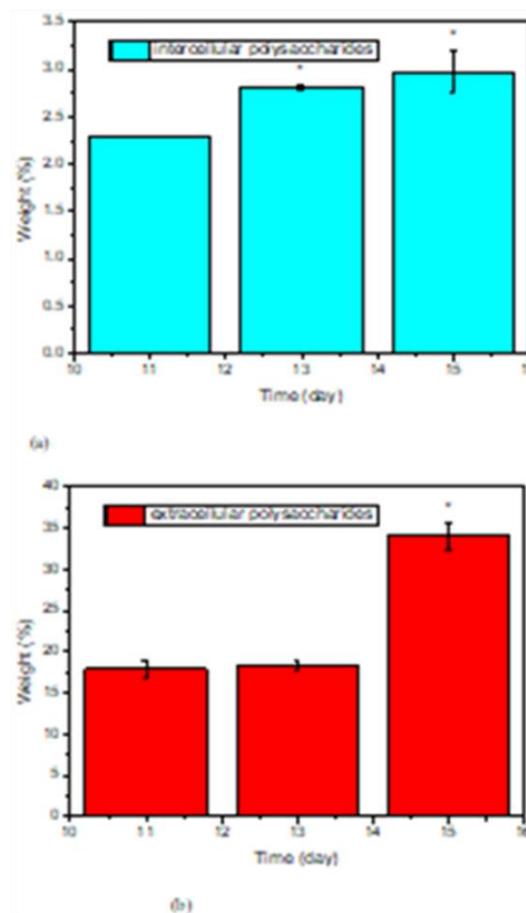


**Fig. 5:** SEM observation of the mycelia pellets of *G. gibbosum* during the liquid fermentation process. (a) Appearance of the pellet after 1 day's cultivation (the right was the magnified image of partial surface, the same below); (b) Appearance of the pellet after 3 day's cultivation; (c) Appearance of the pellet after 5 day's cultivation; (d) Appearance of the pellet after 7 day's cultivation; (e) Appearance of the pellet after 9 day's cultivation

1 day's pellet (Fig. 5b, c and d). From the 9<sup>th</sup> day, it could be seen that the structure of the pellet became more compacted by a large number of the hyphae (Fig. 5e). However, part of the mycelia aggregated on the surface of the pellet became loose because of the exponential growth at this phase, which was consistent with the growth curve. The growth cycle of *G. gibbosum* was relatively longer compared with other species of the genus of *Ganoderma*, such as the liquid fermentation of *G. lucidum* with the pumpkin juice (Zhao *et al.*, 2015).

#### Crude Polysaccharide's Measurement

Fig. 6a shows the variation of intercellular polysaccharide contents of *G. gibbosum* during the liquid submerged fermentation process. As can be seen obviously, when entered the stationary phase after the 11<sup>th</sup> day, with the



**Fig. 6:** The contents of intercellular polysaccharides (a) and extracellular polysaccharides (b) of the mycelia of *G. gibbosum* during the liquid submerged fermentation process. Each histogram is expressed as mean  $\pm$  SD from  $n=3$ . \* Significant difference from  $t=11$  Day ( $P<0.05$ )

decreasing of the mycelia weights (Fig. 4), the contents of the intercellular polysaccharides increasing gradually with the incubation time (Fig. 6a), the content reached a maximum value of  $2.97 \pm 0.22\%$  at the 15<sup>th</sup> day. Similar phenomenon was found in the variation of the extracellular polysaccharide contents (Fig. 6b), the content was up to the biggest value of  $34.12 \pm 1.65\%$  at the 15<sup>th</sup> day. These profiles were consistent with their growth characteristics of the mycelia under the liquid submerged fermentation conditions.

#### Discussion

It's known that the secondary metabolites like antibiotics, toxins are produced by some kinds of microorganisms during the stationary phase. As a kind of secondary metabolites produced by the mycelia, *Ganoderma* polysaccharides are important bioactive substances.

For instance, Zhang *et al.* (2012, 2016, 2017) reported a bioactive extra- and intercellular polysaccharide (PSG)

from *Ganoderma lucidum* and *G. atrum*, which had favourable antioxidant activity. Therefore, it's important to scale-up *Ganoderma* mycelium's cultivation by the liquid submerged fermentation technique.

As the most well-known medicinal edible fungi, *Ganoderma* are popular in traditional Chinese medicine. Based on the unique geographical environment, the bioactivity substances like polysaccharides, ganoderic acid of Huoshan *Ganoderma* are famous for their multiple functions in health. In this work, a kind of wild species Huoshan *Ganoderma*, namely, *G. gibbosum* was successfully cultured with the solid and liquid media from the fruiting body. The growth cycle of the mycelia in the liquid submerged fermentation was about 2 weeks. During the liquid fermentation process, a certain content of the intercellular polysaccharide and extracellular polysaccharide were produced by the mycelia pellets after 11<sup>th</sup> day.

## Conclusion

As the wild species of *Ganoderma*, it's valuable to explore the mycelia cultivation method, especially for the culture collection of the distinctive germplasm resources. In the following work, we will focus on the bioactivities of the polysaccharides and their structural characterization.

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