Hyperproduction of exopolysaccharides by submerged mycelial culture of *Ganoderma lucidum* using a solid seed grown in fine-powder of wheat bran and in vitro evaluation of the antioxidant activity of the exopolysaccharides produced

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Abstract To facilitate *Ganoderma lucidum* submerged culture and obtain high productivity, a fine powder of wheat bran was used to grow the fungus for solid-state fermentation and as solid seed for its submerged cultures. The results indicated that the optimal inoculum size was low, being 0.75 g in 250 mL-sized flasks containing 80 mL medium. The maximal exopolysaccharide concentration and biomass produced was 0.74 and 14.71 g/L, respectively, which is considerably higher than that obtained with the commonly used mycelial pellet liquid seed (0.47 and 8.56 g/L, respectively). The EPS and biomass productivity of the solid seed cultures decreased only slightly, even after a 6-month storage period. EPS produced showed higher antioxidant activity compared with that produced in the liquid seed cultures. The developed solid seed can serve as a ready-to-use inoculum for long-term use in *G. lucidum* submerged culture for the hyperproduction of highly bioactive EPS and biomass.

Keywords *Ganoderma lucidum* · Submerged culture · Solid seed · Exopolysaccharide · Mycelial biomass · Pellet morphology

Introduction

Ganoderma lucidum, a basidiomycete fungus, belonging to the family Polyporaceae, is a well-known traditional Chinese medicinal mushroom. Its wood-textured, fan-like fruiting body, called “Lingzhi” in Chinese, has been widely used to prevent and treat various diseases, such as hepatitis, bronchitis, insomnia, asthma, neurasthenia, and cancer, for centuries in China, Korea, Japan, and other Asian countries [17, 21, 24]. Presently, it is consumed as a popular remedy for minor health disorders and to promote health and longevity in Asia, Europe, and America. Numerous recent studies have revealed that *G. lucidum* has various biological and pharmacological activities, including antitumor [1], antioxidant [20], and immunomodulatory effects [3].

Because of its perceived healthy benefits, there has been a continuous increase in the cultivation for *G. lucidum* in recent years. Currently, the cultivation of *G. lucidum* on sawdust-based artificial logs or broadleaf tree logs is a popular method for its production in China. Using the basidiocarps and their spores as raw materials, diverse value-added *Ganoderma*-based products, like soft capsules, injections, tablets, and drinks can be manufactured. The major disadvantages of this bioprocess for *G. lucidum* production are that a cropping cycle more than 6-month long is usually required [30], and achieving stable product quality is difficult due to the variation in strains and environmental conditions [5, 26]. Thus, alternative production methods are potentially required.

Submerged culture has recently emerged as a promising method for *G. lucidum* production because it has the potential for higher production of biomass and various useful metabolites, in a compact space and less time with less chance of contamination [31]. Owing to these

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advantages, the submerged culture of *G. lucidum* for the production of polysaccharides, triterpenoids, or mycelial biomass has been studied, focusing mainly on optimization of process parameters [11], development of novel fermentation strategies, such as elicitation [33], and manipulation of genetic materials [29]. As the isolation of exopolysaccharide (EPS) from fermentation broth is much easier compared with that of the polysaccharides from fruit bodies and mycelia [18], EPS production has received growing attention over the past few years.

In an overview of previous studies, we found that presently, mycelial liquid seed is the only inoculum used for *G. lucidum* submerged cultures. However, most mushroom species, including *G. lucidum* grow slowly, both in slants and liquid media; thus, preparation of such liquid seed from slant stock culture is time-consuming, often requiring more than 10 days. Moreover, there is a high risk of microbial contamination due to multiple-round seed transfers during the preparation. Another disadvantage of the liquid seed is that *G. lucidum* mainly grows in the pellet form in liquid culture, resulting in fewer hyphal growing tips in this seed, which considerably affects the process productivity of the culture after inoculation into medium.

In the present study, with the aim of facilitating submerged culturing of *G. lucidum* both in research study and for industrial purposes for the efficient production of EPS and mycelial biomass, we attempted to develop a solid seed of *G. lucidum*, and evaluated EPS production in its submerged cultures. In vitro antioxidant activity of the EPS produced was also determined. Moreover, to establish a reference for the developed inoculum as a ready-to-use seed used in submerged cultures of *G. lucidum* for a long term period, the change in the fermentation productivity of the developed seed during storage was determined. This study provides an alternative inoculum for effective submerged fermentation of *G. lucidum*.

Materials and methods

Strain and media

G. lucidum G10016 was obtained from the Mycological Research Centre, Fujian Agriculture and Forestry University. The seed medium used was composed of the following components (g/L): glucose, 30; peptone, 4; yeast extract, 2.5; KH_2PO_4 , 1.0; MgSO_4 , 0.25; and vitamin B₁, 0.1. Fermentation medium contained (g/L): glucose, 35; peptone, 5; yeast extract, 2; KH_2PO_4 , 1.0; and MgSO_4 , 0.5. Both media were autoclaved at 121 °C for 20 min.

Preparation of solid seed

A fine powder (60 mesh) of wheat bran was utilized, tap water was added to adjust a moisture content of 58%. The substrate (400 g) was transferred to a 1000 mL flask, and slightly packed. After sterilized and cooled to room temperature, the flask was inoculated with one piece of mycelial agar disc (10 mm in diameter) cut from the advancing margin of actively-growing 6-day-old cultures in PDA plates, and incubated at 25 °C in the dark. When the substrate was completely penetrated by the mycelium, it was used as solid seed for submerged cultures, or stored at 4 °C for subsequent use.

Production of liquid seed

G. lucidum was inoculated into PDA slants, and incubated at 25 °C for 7 days to obtain actively growing mycelium. For the first pre-culture, the freshly grown slants were squashed with an inoculated rake, and a half of them were transferred into a 250 mL flask containing 80 mL seed medium, and incubated at 28 °C for 4 days with shaking at 160 rpm. For the second pre-culture, the first pre-culture (5 mL) was inoculated into 75 mL seed medium, and cultured for 2 days. It served as a pellet-type liquid seed used in submerged cultures. To obtain a more homogenous liquid seed, the second pre-culture was homogenized in a polytron (IKA, Tltra-turrax, Germany) at 10,000 rpm for 30 s, and used as a homogenized-mycelia liquid seed.

Submerged culture

To determine the optimal inoculum size of the solid seed for the initiation of submerged cultures, each 250-mL flask containing 80 mL fermentation medium was inoculated with a seed amount varying from 0.25 to 1.5 g, and the biomass and EPS produced were measured at the end of cultivation. After optimization, the cultures inoculated with the solid seed, homogenized-mycelia liquid seed, or pellet-type liquid seed were grown in 250-mL flasks containing 80 mL medium for comparing the fermentation performance of the seeds. For the solid seed, the inoculum size was 0.75 g per flask and 5% (v/v) (a commonly used inoculum size) for the pellet-type liquid seed. For the homogenized-mycelia liquid seed, the inoculum size was 5% (v/v) as an instance with high inoculum density, because it contained large amounts of hyphal fragments. All flasks were incubated at 28 °C for 7 days with shaking at 160 rpm.

Measurement of pellet size and counting

Pellet size was determined using a ruler with 0.5-mm graduations. At least, more than 100 pellets for each sample were randomly chosen for measurement, and the average size was calculated. Pellets were counted by transferring 1 mL sample from the cultures at regular intervals of 24 h into a 9-cm Petri dish that contained 2 mL distilled water for better enumeration.

Measurement of mycelial biomass and EPS

To measure the mycelial biomass accurately, three flasks were taken each time, and the biomass in each flask was isolated by centrifugation at $8000 \times g$ for 10 min, followed by washing the precipitated mycelia three times with distilled water and drying at 60°C until a constant weight was obtained. In particular, for cultures of the solid seed inoculum, the biomass content was calculated by subtracting the dry weight of the solid seed from the measured dry weight. The biomass of solid seed was determined using the method described by Economou et al. [8]. For EPS determination, samples were prepared, as described by Fang et al. [9], and the concentration was determined using the phenol–sulfuric acid method [7].

Preparation of EPS

The EPS was assayed as follows: the crude EPS in supernatants was precipitated by the addition of four times the volume of 95% (v/v) ethanol, vigorous mixing, and overnight incubation at 4°C . The insoluble components were harvested by centrifugation at $8000 \times g$ for 10 min, and washed with 75% ethanol. The resultant precipitate was lyophilized to obtain the *G. lucidum* EPS.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was measured according to the method of Winterbourn and Sutton [28]. The reaction mixture contained 1 mL 0.15 M phosphate buffer saline (pH 7.4), 1 mL 40 $\mu\text{g/mL}$ safranin, 1 mL 0.945 mM EDTA- Fe^{2+} , 1 mL 3% (v/v) H_2O_2 , and 0.5 mL the EPS (0.1–1 mg/mL). After incubation at 37°C for 30 min, the absorbance of EPS was measured at 560 nm. Vitamin C was used as a positive standard. The hydroxyl radical scavenging activity was expressed as the following equation:

$$\text{Scavenging rate}(\%) = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of EPS.

DPPH radical scavenging activity assay

The DPPH radical scavenging activity was measured according to the method of Blois [4], with minor modifications. Briefly, 2 mL of 0.2 mM DPPH in 95% ethanol was added to 2 mL of sample solution at different concentrations (0.1–1 mg/mL). After vortexing, the mixture was kept in the dark at room temperature for 10 min, and centrifuged at $6000 \times g$ for 10 min. The absorbance of the supernatant was measured at 517 nm. The control was done with ethanol instead of DPPH, while ethanol was used as the blank. Vitamin C was used as a positive standard. DPPH radical scavenging activity was calculated according to the following equation:

$$\begin{aligned} \text{DPPH radical scavenging activity}(\%) \\ = \left(1 - \frac{A_s - A_c}{A} \right) \times 100 \end{aligned}$$

where A_s is the absorbance of the reaction solution, A_c is the absorbance of the ethanol control solution, and A is the absorbance of the solution including 2 mL DPPH and 2 mL ethanol.

Results and discussion

Effect of inoculum size of the solid seed on the production of EPS and mycelial biomass

In microbial fermentation, a suitable inoculation method can not only enhance metabolite production, but also simplify the commercial fermentation process [32]. To our knowledge, reports on the use of solid seed in the submerged culture of edible and medicinal mushrooms are not available. For varying solid seed inocula, the data obtained for biomass and EPS production, residual glucose levels, and pH at the end of the culture period for flask-submerged cultures of *G. lucidum* are listed in Table 1. The biomass production increased with the increasing inoculum size up to 1.25 g/flask, with the corresponding maximal biomass produced being 15.34 g/L. EPS production increased with the increase in inoculum size up to 0.75 g/flask, with the maximal EPS concentration being 0.76 g/L and the corresponding biomass produced being 14.46 g/L. A further increase did not result in an increase in EPS production. It has been previously reported that the inoculum size affects EPS production [9]. Regarding glucose utilization, the consumption of glucose increased with the increase in inoculum size in the test range. Final pH values of culture broths varied between 3.72 and 4.12. As the production of EPS was a major focus of this work, the optimal inoculum size was, therefore, determined to be 0.75 g/flask under the current experimental conditions.

Table 1 Results of *G. lucidum* submerged mycelial cultures in 250 mL flasks with different inoculum sizes of the developed solid seed

Inoculum size (g/flask)	Biomass (g/L)	EPS (g/L)	Final pH	Residual glucose (g/L)
0.25	7.44 ± 0.71	0.41 ± 0.05	3.94 ± 0.11	21.22 ± 0.94
0.50	10.23 ± 0.52	0.53 ± 0.06	3.89 ± 0.11	18.12 ± 0.42
0.75	14.46 ± 0.43	0.76 ± 0.07	3.72 ± 0.08	15.48 ± 0.79
1.00	15.13 ± 1.22	0.71 ± 0.08	4.12 ± 0.14	12.34 ± 0.45
1.25	15.34 ± 0.84	0.75 ± 0.04	3.83 ± 0.07	10.26 ± 0.81
1.50	15.17 ± 0.67	0.68 ± 0.05	3.96 ± 0.12	9.77 ± 0.78

Data are mean ± standard deviation of three independent flask cultures, at the end of the cultivation

Submerged culture of *G. lucidum* inoculated with the solid seed and the other two types of seed

Comparison of time profiles of process parameters for the production of EPS and biomass

It is well-known that mycelial liquid seed is commonly used for submerged cultures of *G. lucidum*. In our previous study, to increase the number of hyphal growing tips, mycelial pellets of *G. lucidum*, used as an inoculum, were homogenized, prior to use; this homogenate was extremely suitable for use in submerged fermentation, and the production of biomass and EPS was significantly improved [19]. The time profiles of flask cultures using the solid seed, homogenized-mycelia liquid seed (served as a test with high inoculum density), and pellet-type liquid seed are shown in Fig. 1.

As indicated in Fig. 1, when homogenized-mycelia liquid seed was used for inoculation, the mycelial growth was the fastest, and a maximal biomass of 16.32 g/L was obtained on day 5. For solid seed, the growth was slow before day 1, but thereafter, rapid growth was observed, and a maximal biomass of 14.71 g/L was recorded on day 5. For the pellet-type liquid seed, the fermentation rate was slow, with a maximal biomass of only 8.56 g/L obtained on day 6. The higher biomass production by the homogenized-mycelia liquid and solid seed cultures can be attributed to the formation of high concentration of small pellets, since the diffusion of nutrients, particularly oxygen, into the center of the pellets becomes easier for small pellets. It has been reported previously that small sized pellets facilitate high biomass production [9, 25].

Figure 1(B) shows that when the solid seed and homogenized-mycelia liquid seed are used in cultures, the EPS production increased with time up to day 5, with EPS concentration being 0.74 and 0.84 g/L, respectively, and a slight decrease was observed, thereafter. It has been previously reported that a prolonged cultivation time can lead to a reduction in EPS production [10]. For the pellet-type liquid seed, the EPS production increased up to day 6, and EPS concentration was 0.47 g/L. The reason for higher EPS production by the solid and homogenized-mycelia

liquid seed cultures is due to the higher biomass obtained, since EPS is partly a growth-associated product [30]. The obtained yield was lower than that reported by Zhu et al. [33], who reported an EPS concentration of 0.92 g/L, but significantly higher than that reported by Chang et al. [5], who reported a 0.42 g/L EPS concentration. These discrepancies may be largely due to the difference in strains, culture conditions, and analytical methods used.

As shown in Fig. 1(C), the glucose in the broth was utilized quickly by the solid seed and the homogenized-mycelia seed cultures, while the pellet-type seed cultures utilized glucose slowly. At the end of the culturing period, glucose utilization was the highest in cultures with the homogenized-mycelia liquid seed inoculum, and the lowest in the pellet-type liquid seed cultures. A positive correlation was observed between glucose utilization and biomass formation. Figure 1(D) shows the change in pH of the three cultures with different seeds during cultivation. The pH of the broth decreased rapidly at the initial stage, and then there was not much change during the middle and later phases for all the test seeds. Similar pH kinetics have also been reported for *G. lucidum* liquid fermentation cultures [10]. At the end of culture period, the pH values were similar among the three different cultures.

Comparison of time profiles of pellet number and size in cultures

The morphological properties of filamentous fungi in submerged culture have been shown to play a critical role during industrial fermentation and commercial production of many metabolites [2, 22]; the morphological properties are often characterized by mean pellet diameter, circularity, and roughness [27]. As shown in Fig. 2(A), the pellet concentration was highest on day 1 in cultures inoculated with the solid seed, immediately followed by those inoculated with the homogenized-mycelia liquid seed, while the cultures inoculated with the pellet-type liquid seed showed the lowest concentration of pellets. Between day 1 to day 2, there was a decrease in pellet concentration in cultures with the solid seed and homogenized-mycelia liquid seed inoculum. It was possibly due to aggregation of

◀**Fig. 1** Comparative profiles of submerged cultures of *G. lucidum* in flasks inoculated with the solid seed (closed circles), homogenized-mycelia liquid seed (half-closed circles), and pellet-type liquid seed (open circles)

Fig. 2 Time profiles of pellet number and size during submerged cultures of *G. lucidum* in flasks inoculated with the solid seed (closed circles), homogenized-mycelia liquid seed (half-closed circles), or pellet-type liquid seed (open circles). The pellet size was determined after 24 h of cultivation for cultures inoculated with the solid seed and homogenized-mycelia liquid seed

few small pellets and mycelial aggregates. Thereafter, the pellet concentration gradually increased till the end of cultivation, with the concentration being 430.31 number/mL for the homogenized-mycelia seed and 393.56 number/mL for solid seed cultures. For the pellet-type inoculated cultures, pellet concentration increased from the inoculation time to the end of the cultivation, with a concentration of only 186.66 number/mL. The considerable difference in pellet concentration among the three cultures was possibly

due to the difference in the number of hyphal growing points present in the initial inoculum.

In Fig. 2(B), it is seen that the pellets became larger in size with increasing cultivation time up to day 6 for all cultures; thereafter, a slight decrease in pellet size with time was observed. The reduction in the pellet size during the late fermentation stages may be due to the loss of the outer hairy parts of the pellets. A similar observation has been reported in submerged fermentation of various mushroom species [16, 23]. The size of the largest pellet was 3.72 mm (diameter); such large sized pellets were observed in the pellet-type liquid seed cultures. The pellet diameter for the homogenized-mycelia liquid seed was 2.63 mm, and 3.14 mm for the solid seed.

Comparison of the proportion of pellets of different sizes among cultures and their corresponding morphology

As shown in Fig. 3(A), the pellets with a diameter of 2–3 mm accounted for 53.28% of the total number of pellets in cultures inoculated with the solid seed, while pellets with a diameter of 1–2 mm accounted for 25.14%. The number of small (< 1 mm diameter) and large (> 4 mm diameter) pellets was low and was only 4.45 and 3.37%, respectively. For cultures inoculated with the homogenized-mycelia liquid seed (Fig. 3(B)), the pellets with diameters of 1–2 and 2–3 mm accounted for 42.67 and 40.64% of the total number of pellets, respectively, and the number of both small (< 1 mm diameter) and larger

large (> 4 mm diameter) pellets was low. Conversely, for the cultures inoculated with the pellet-type liquid seed (Fig. 3(C)), a high proportion (51.31%) of big pellets (more than 3 mm in diameter) was observed, but the percentage of small pellets (< 1 mm diameter) was low, 2.66%.

The pellet morphology differed considerably in the cultures with the three different seed inocula. The pellets with compact central cores, fluffy outer zones, and moderate size were observed in cultures with the solid seed, while compact smooth small pellets were formed for homogenized-mycelia liquid seed cultures; thick-branched big pellets were produced in pellet-type liquid seed cultures. The significant variations in pellet size and morphology in cultures with the three different seeds could be attributed to the difference in the inoculation density. A similar observation has also been reported previously [6, 9].

Changes in the EPS and biomass productivity of the solid seed during storage

The changes in EPS and biomass productivity of the solid seed during a storage period of 6 months were examined in flask submerged cultures. The results in Fig. 4 indicated that the production potential of EPS and biomass remained almost unchanged up to a storage period of 3 months. At this time, the biomass was 14.36 g/L, which amounted to 96.96% of that obtained with the original seed (14.81 g/L). As for EPS production, it was also desirable as high as 0.76 g/L, which was 92.68% of that obtained with the original seed (0.82 g/L). Thereafter, a slightly decreasing trend in EPS and biomass production was observed, and at 6 months of storage, the production of biomass (13.67 g/L)

Fig. 3 Distribution of pellet diameter measured in cultures with the solid seed (A), homogenized-mycelia liquid seed (B), and pellet-type liquid seed (C) inocula, at 7 days of cultivation

Fig. 4 Changes in EPS and biomass production in submerged flask cultures using the developed *G. lucidum* solid seed, during a 6-month storage period

and EPS (0.73 g/L) was equal to 92.30 and 89.02%, respectively, of that obtained from the original seed. It can be concluded, that within our test range, the length of storage time of the solid seed did not affect its productivity significantly. Therefore, the developed solid seed can be used for a long period as a ready-to-use seed for its submerged cultures. Taken together, the solid seed is superior to mycelial pellet liquid seed for use in submerged cultures of *G. lucidum* for the production of EPS and biomass. In our view, preparing this type of solid seed is also likely to be suitable for other mushrooms for use in their submerged cultures for the production of EPS and biomass.

Antioxidant activities of EPS produced from three different types of seed cultures

It has been reported that the antioxidant activities of polysaccharides play an important role in preventing and

curing many human diseases; therefore polysaccharides can be studied as novel potential antioxidants [15]. Antioxidant activities of chemicals and natural products have been attributed to various reactions and mechanisms, including radical scavenging, reductive capacity, prevention of chain initiation, binding of transition metal ion catalysts [12]. There have been extensive investigations and reports on the antioxidant activity of EPS of edible and medicinal mushrooms [13, 14], but reports on the antioxidant activity of EPS of *G. lucidum* are rare.

As shown in Fig. 5, the hydroxyl radical and DPPH scavenging activities of the EPS from the three cultures increased with the increase in sample concentration in a test range of 0.1–1.0 mg/mL; the antioxidant activities increased in a dose-dependent manner, but they were weaker than those of vitamin C for each test concentration. It was interesting to note that EPS produced by the solid seed cultures showed similar antioxidant activity to that of EPS produced by the homogenized-mycelia liquid seed culture, but significantly higher than that of EPS produced by the pellet-type liquid seed cultures under the same concentration. The difference in their antioxidant ability may be related to the variation in the monosaccharide composition, content of hydroxyl and carboxy groups, and hydrogen donation ability. To elucidate the underlying mechanisms for this difference, a detailed study on the chemical properties and structural characteristics of the three types of EPSs is potentially required.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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Fig. 5 Hydroxyl radical (A) and DPPH (B) scavenging activity of the EPS produced by the three different inocula (filled circles, solid seed; half-closed circles, homogenized-mycelia liquid seed; and open circles, pellet-type liquid seed) with vitamin C (open squares) as a positive control

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