Optimization of submerged culture conditions involving a developed fine powder solid seed for exopolysaccharide production by the medicinal mushroom *Ganoderma lucidum*

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Abstract To facilitate *Ganoderma lucidum* submerged cultivation and achieve high productivity, four fine powder solid substrates incorporated with different nitrogen-rich supplements were utilized to grow the fungus and as solid seed for its submerged culture. Of the four solid seeds, the soybean meal solid seed gave the highest biomass (10.73 g/L) and exopolysaccharide (EPS) (1.22 g/L), higher than those (8.36 g/L biomass and 0.44 g/L EPS) obtained with mycelial liquid seed. The optimal level of soybean meal supplementation was 20% (w/w) for production of the solid seed. Following single factor experiments, levels of three selected process variables were optimized as: the moisture content of solid seed, 70%; inoculum size, 0.8 g/flask; and rotary speed, 160 rpm. These conditions were validated experimentally with improved EPS yield of 1.33 g/L. The developed solid seed can be conveniently used for *G. lucidum* submerged culture with improved EPS productivity.

Keywords *Ganoderma lucidum* · Submerged culture · Solid seed · Exopolysaccharide · Response surface methodology

Introduction

Ganoderma lucidum, commonly known as Lingzhi in China, is one of the most famous traditional Chinese medicinal mushrooms. Its fruiting body has been widely used as a folk remedy to treat various diseases such as hepatitis, bronchitis, insomnia, asthma, neurasthenia, and various cancers in oriental countries (Jong and Birmingham, 1992; Mizuno et al., 1995). In addition to its use for medicinal purposes, nowadays *G. lucidum* is also popularly consumed as a dietary supplement to promote health and longevity. Until now, a large number of bioactive components have been isolated from *G. lucidum*, and the polysaccharides and triterpenoids are the primary functional components, showing various physiological activities such as antitumor, antioxidant, and immunomodulatory effects (Shiao, 2003).

The wild fruiting body of *G. lucidum* is rarely found in nature. To meet the escalating demand of *G. lucidum*, it is commonly cultivated on sawdust-based artificial logs or broadleaf tree logs to obtain its fruit bodies. However, it usually takes several months to harvest its fruiting body (Yang et al., 2000), constituting a major obstacle for its industrial production. Moreover, there is also a considerable variation in product quality because of differences in the strains utilized and in the environmental conditions for cultivation (Chang et al., 2006; Song et al., 1998), which makes it extremely difficult to be commercially developed as a high-quality, standardized health food and medicine. As such, developing or adopting alternative methods for production of *G. lucidum* biomass, polysaccharides, or triterpenoids are potentially required.

Submerged culture has shown its advantage for higher mycelial production due to a shorter cultivation period with less space requirement, and it also allows for the easy

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control of contamination (Bae et al., 2000). Owing to these advantages over traditional solid cultivation, many attempts have been focused on submerged fermentation of mushrooms to produce useful metabolites such as ergothioneine (Tepwong et al., 2012). Among various mushroom species, *G. lucidum* has attracted special attention for production of polysaccharides and triterpenoids because of their remarkable biological and pharmacological activities. These mainly focus on strain screening (Wei et al., 2014), optimization of medium compositions (Chang et al., 2006), and development of novel bioprocesses such as elicitation (Zhu et al., 2008).

During microbial fermentation, the inoculation method is significant in enhancing metabolite production and simplifying the process (Li et al., 2015; Yen and Chiang, 2015). For submerged culture of mushrooms, mycelial liquid seed is commonly adopted (Fang and Zhong, 2002; Tepwong et al., 2012). However, as most mushroom species, including *G. lucidum*, grow slowly both in slants and liquid media, the time needed for preparing such a liquid inoculum is rather long, often more than 10 days. Moreover, microbial contamination frequently occurs, in great part due to the requirement of multiple-round seed transfers during the process and the slow mycelia growth. Finally, as *G. lucidum* typically grows as large and compact pellets in liquid culture (Yang et al., 2009), this mycelial culture has fewer hyphal growing tips. This feature considerably affects process productivity when it was used as liquid inoculum. To overcome this drawback, these mycelial pellets and/or hyphal aggregates are usually homogenized before being used as inocula (Upadhyay et al., 2014; Yang et al., 2009). In our previous study, as an alternative inoculation method, a solid seed of *G. lucidum* grown on wheat bran fine powder was developed and exhibited promising results for exopolysaccharide (EPS) production during submerged fermentation (Liu and Zhang, 2018).

During submerged cultivation of *G. lucidum*, a wide variety of process variables, including inoculation density, temperature, initial medium pH, and rotary speed, are known to significantly affect cultivation performance (Fang and Zhong, 2002; Fang et al., 2002; Yang and Liao, 1998). For solid state fermentation, substrate particle size, moisture content, inoculum size, nitrogen source, etc. have been proved to be crucial factors influencing metabolite production (Rocky-Salimi and Hamidi-Esfahani, 2010; Vu et al., 2010; Yang et al., 2014). The major objective of the present study is to develop a solid seed of *G. lucidum* on a fine powder sawdust-based substrate suitable for use in its submerged culture. In order to provide basically scientific knowledge on better utilization of this type of inoculum and maximize EPS productivity, submerged culture conditions were also optimized using response surface methodology (RSM). RSM is an effective statistical

method that can design experiments, evaluate the effects of factors, build mathematical models, and determine optimum conditions of factors for desirable responses (Montgomery, 2000). Box–Behnken design (BBD) is a type of RSM and suitable for fermentation optimization (Jia et al., 2015), and frequently used in experimental design for RSM (Reddy et al., 2008; Zhao et al., 2009). The solid seed developed in this work will help promote studies on submerged culture of *G. lucidum*, and has a promising application prospect in industrial fermentation.

Materials and methods

Strain and maintenance

Ganoderma lucidum G10016, obtained from the Mycological Research Centre, Fujian Agriculture and Forestry University, Fuzhou, China, was used. This fungus grows fast during solid cultivation, and produces fruiting bodies (mature) with reniform and convex shape, and reddish color. It was grown on potato dextrose agar (PDA) slants for 7 days at 25 °C, stored at 4 °C, and sub-cultured very month.

Media

Seed culture medium was composed of (g/L): glucose 20, yeast extract 5, peptone 3, KH_2PO_4 1, MgSO_4 0.5, and vitamin B₁ 0.05. For submerged cultivation, the medium contained (g/L): glucose 35, peptone 3, yeast extract 2, KH_2PO_4 1, and MgSO_4 0.5. Both media were autoclaved at 121 °C for 20 min.

Liquid seed preparation

Ganoderma lucidum was inoculated and incubated in PDA slants at 25 °C for 7 days. They were then squashed with an inoculated rake, and a half of them were inoculated into a 250 mL flask containing 80 mL seed medium. Cultivation was run at 28 °C for 4 days with shaking at 160 rpm. Next, 5 mL of this culture was inoculated into 80 mL seed medium dispensed in a 250 mL flask, cultured further for 2 days, and used as mycelial liquid seed.

Development of solid seed

Four supplements, including wheat bran, rice bran, soybean meal, and corn flour, were utilized as nitrogen source in substrate formulation by using sawdust as a basal ingredient. Before use, wheat bran, rice bran, soybean meal, and sawdust were separately ground in a mill. The ground ingredients as well as corn flour were separately screened

through a 40-mesh sieve to obtain a fine powder. Each of the four test supplements at 20% (w/w) level by dry weight was individually added to the fine powder sawdust, mixed, and adjusted to 60% moisture content. Moistened substrate (100 g) was lightly packed into 250 mL flasks, cotton plugged, and autoclaved for 30 min at 121 °C. After being cooled, an agar plug of 10 mm diameter cut from the periphery of an actively growing mycelial colony in a 9 cm Petri dish was inoculated into the flasks. After incubated at 25 °C for 7 days, the solid cultures were utilized as solid seed for use in *G. lucidum* submerged cultivation.

Submerged culture conditions

All submerged cultures were carried out in 250 mL flasks with 80 mL fermentation medium for a cultivation period of 6 days. For screening of a better solid seed (60% moisture content) and determining optimal addition level of soybean meal, inoculum size 0.6 g (wet weight)/flask, temperature 28 °C, natural initial pH (around 6.0), and rotary speed 160 rpm were set for submerged culture. Flasks inoculated with 10% (v/v) of the mycelial liquid seed were run as the control for comparison. For optimization studies using one-factor-at-a-time and RSM methods, sets of tested process variables varied with the experimental design. The mycelial growth and/or EPS yield were assayed at the end of cultivation. All flask culture experiments were carried out in triplicate, and the data are reported as the mean $\pm$ standard deviation or as average values.

Single factor experiment for preliminary optimization

The effect of initial moisture content (50–90%) of soybean meal supplemented substrates utilized for solid seed preparation (referred to as the moisture content of solid seed afterwards, without considering the water evaporation during the growth stage), inoculum size (0.2–1.2 g), rotary speed (120–200 rpm), temperature (24–32 °C), and initial medium pH (3.0–7.0) on mycelial growth and EPS production was investigated using one-factor-at-a-time approach. Herein, to ensure the accuracy, the required initial medium pH values were adjusted after medium sterilization with either autoclaved 1 mol/L NaOH or 1 mol/L HCl.

Response surface optimization of EPS production

A Box–Behnken design (BBD) (Box and Behnken, 1960) was used in the optimization of EPS production. Three process variables including the moisture content of solid seed, inoculum size, and rotary speed were chosen as

independent variables and designated X_1 , X_2 , and X_3 , respectively. The low, intermediate and high levels of each selected variable were designated as -1 , 0 , and $+1$, respectively, given in Table 1. The average of EPS concentration obtained was taken as the response. This BBD design with a number of 17 experimental runs was performed randomly shown in Table 2. The obtained results were fitted to the second-order quadratic polynomial equation as below:

$$Y = \beta_0 + \sum_{j=1}^k \beta_j x_j + \sum_{j=1}^k \beta_{jj} x_j^2 + \sum_{i < j} \beta_{ij} x_i x_j \quad (1)$$

where Y is the EPS yield, β_0 , β_j , β_{jj} and β_{ij} are the regression coefficients for intercept, linearity, square and interaction, respectively, while x_i and x_j are the coded variables. For statistical analysis, the variables were coded based on the following equation:

$$x_i = (X_i - X_0) / \Delta X \quad (2)$$

where x_i is the coded value of the variable X_i , X_0 is the actual value of X_i at the centre point, and ΔX is the value of step change of variable. Design-Expert (Version 7.0) software package was used to analyze the experimental data. The statistical significance of the polynomial model equation was carried out by an Fischer's F -test and the significance of the coefficients was tested by t tests. The p values of less than 0.05 were considered to be statistically significant. The variance explained by the model is given by the coefficient of determination, R^2 .

Measurement of biomass

Biomass was estimated as described by Feng et al. (2016). At the end of cultivation, three flasks as three repetitions were taken for each analysis. The biomass content in each flask was separated 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 achieved. The net biomass was calculated by subtracting the dry weight of the solid seed utilized from the measured dry weight.

Assay of EPS formation

Samples (5 mL) were withdrawn from the culture broth after mycelia separation. 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 dissolved with distilled water (60 °C) to a constant volume of 100 mL. The resultant

Table 1 Code and level of process variables used for Box–Behnken design

Variables	Symbols		Coded levels		
	Uncoded	Coded	− 1	0	1
Solid seed moisture content (%)	X_1	x_1	50	70	90
Inoculum size (g/flask)	X_2	x_2	0.6	0.8	1.0
Rotary speed (rpm)	X_3	x_3	140	160	180

Table 2 Box–Behnken design matrix of the three process variables in coded units along with experimental values of EPS production by *G. lucidum*

Runs	x_1 : solid seed moisture content	x_2 : inoculum size	x_3 : rotary speed	EPS (g/L)
1	0	0	0	1.26
2	1	0	1	0.83
3	0	0	0	1.28
4	0	0	0	1.31
5	− 1	− 1	0	0.91
6	1	0	− 1	0.76
7	− 1	0	− 1	0.93
8	− 1	0	1	0.81
9	1	− 1	0	0.67
10	0	1	1	0.97
11	0	0	0	1.25
12	1	1	0	1.03
13	0	− 1	− 1	0.62
14	0	1	− 1	1.13
15	0	0	0	1.34
16	0	− 1	1	1.08
17	− 1	1	0	1.10

solution was used for EPS assay using the phenol–sulfuric acid method (Dubois et al., 1956).

Results and discussion

Screening of a suitable supplement for *G. lucidum* solid seed preparation for submerged culture and determining its optimal addition level

Supplementation of substrates with nitrogen-rich additives is commonly adopted for obtaining high fruiting yield and improving quality during mushroom cultivation (Moonmoon et al., 2011; Naraian et al., 2009). To develop an effective and economical solid seed suitable for use in submerged culture of *G. lucidum* for EPS production, four supplements were examined as nitrogen sources for substrate formulation with sawdust as a basal ingredient. As shown in Fig. 1A, with the four solid seeds for the submerged cultivation, the soybean meal solid seed gave the highest biomass at 10.73 g/L, followed by wheat bran solid seed (10.15 g/L), corn flour solid seed (9.56 g/L), and rice bran solid seed (8.87 g/L). These obtained biomass yields were significantly higher than that of the control

(8.36 g/L). Regarding EPS formation, the highest yield of 1.22 g/L was also achieved by the soybean meal solid seed, while rice bran solid seed had the lowest yield of 0.74 g/L, but still higher than that of the control (0.44 g/L).

The higher yield of biomass and EPS obtained for the four test solid seeds over the liquid seed may be attributed to the formation of a high amount of hyphal growing points in cultures, resulting from the dissociation of inoculated solid seed into numerous mycelia-covered tiny particles. In literature, high inoculation density leading to a high production of EPS, ganoderic acid, and biomass has been reported in *G. lucidum* submerged fermentation (Fang et al., 2002). Based on this, the above explanation for the high yields of biomass and EPS associated with the solid seed is reasonable.

With both biomass formation and EPS yield as indexes, of the four tested supplements, the use of soybean meal is suitable for the production of *G. lucidum* solid seed. The better performance of soybean meal over other supplements for solid seed production could be attributed to high content of protein and residual oils present in it. Protein is a nitrogen source that favors mycelial growth and vegetable oils stimulate mycelial growth of *G. lucidum* in both submerged and solid state fermentations (Feng et al., 2017;

Fig. 1 Effect of different supplement-contained solid seeds (A) of *G. lucidum* and soybean meal addition level (B) on mycelial growth and EPS production

Postemsky et al., 2014). As such, better mycelial growth could be attained, implying an improvement in the quality of solid seed and therefore, improved fermentation performance.

To determine optimal supplement level, soybean meal addition varying between 10 and 50% (w/w) in substrates was examined. As is evident from Fig. 1B, both mycelial growth and EPS production increased with increasing soybean meal supplementation in solid seed from 10 to 20% (w/w). A further increase caused a decline in both yields. This may be attributed to the excess of nitrogen source in the substrate, leading to a negative effect on mycelial growth and its vitality, and thus, a poor quality of solid seed. High dose of nitrogen source in substrates leading to poor mycelial growth and low fruiting yield has been demonstrated by several researchers (Moonmoon et al., 2011; Naraian et al., 2009). Thus, the soybean meal addition level at 20% (w/w) was used in subsequent studies for solid seed production.

Preliminary optimization of main process variables by one-factor-at-a-time approach

Effects of the moisture content of solid seed

Initial moisture content is a critical factor influencing microbial growth and biosynthesis of enzymes during solid state fermentation (Vu et al., 2010; Yang et al., 2014). Thus, it is reasonable to hypothesize that the initial moisture content of substrate utilized for *G. lucidum* solid seed preparation may affect its quality and therefore its fermentation potential. The effect of moisture content of

soybean meal solid seed on mycelial growth and EPS production was investigated under the condition of inoculum size 0.6 g/flask, rotary speed 160 rpm, temperature 28 °C, and natural initial pH (around 6.0). As shown in Fig. 2A, both mycelial growth (around 10.50 g/L) and EPS production (approximately 1.20 g/L) were maximal when the moisture content of solid seed was at 60% and 70%. Out of this range, their production decreased. Hence, the optimal moisture content of solid seed was between 60 and 70% for biomass and EPS production. It has been reported that during solid state fermentation, high moisture levels lead to decreased substrate porosity as well as reduced oxygen transfer, but low moisture levels lead to poor growth and poor accessibility to nutrients (Lonsane et al., 1985; Pandey, 1992). Thus, our results are generally in agreement with their findings. In subsequent single factor tests, the soybean meal solid seed with 70% moisture content was prepared and used.

Effects of inoculum size

Inoculum size significantly influencing mycelial growth and EPS production in submerged fermentation of mushrooms has been well documented (Fang et al., 2002; Le et al., 2007). The effect of inoculum size of the soybean meal solid seed on mycelial growth and EPS production was investigated by holding rotary speed at 160 rpm and temperature at 28 °C, with natural initial pH (around 6.0). As shown in Fig. 2B, mycelial growth increased rapidly when inoculum size increased from 0.2 g to 0.6 g/flask with the correspondingly rapid increase of biomass from 5.16 to 10.43 g/L, and then slightly increased to 11.25 g/L

Fig. 2 Effect of the moisture content of solid seed (A), inoculum size (B), rotary speed (C), temperature (D), and initial pH (E) on mycelial growth and EPS production by *G. lucidum*

at 0.8 g/flask. A further increase did not obviously improve mycelial growth. The production of EPS gradually increased with increasing inoculum amount from 0.2 g to 0.8 g/flask, with a corresponding increase from 0.62 to

1.26 g/L, beyond which a decreasing trend was observed. This result is somewhat different from that of Fang et al. (2002), where high inoculation density favored EPS production during *G. lucidum* submerged cultivation. The

optimal inoculum size under the current experimental conditions employed was 0.8 g/flask for EPS production. This level was also acceptable for mycelial growth.

Effects of rotary speed

Rotary speed has a significant effect on mycelial growth and EPS production by *Ganoderma* strains during submerged fermentation (Kim et al., 2006a; Yang and Liao, 1998). Using the soybean meal solid seed in the submerged cultivation, the effect of rotary speed on mycelial growth and EPS production was determined under conditions: inoculum size 0.8 g/flask, temperature 28 °C, and natural initial pH (around 6.0). As shown in Fig. 2C, both mycelial growth and EPS production increased as rotary speed increased up to 160 rpm, with maximal yields of 11.28 g/L and 1.24 g/L, respectively. Higher or lower than 160 rpm levels led to a decrease in their production. Hence, rotary speed at 160 rpm was desirable for both mycelial growth and EPS production when the solid seed was used. This result is generally in accordance with the findings of several previous reports, where the optimum for EPS production by *G. lucidum* was 150 rpm (Yang and Liao, 1998) and 160 rpm by *Pleurotus nebrodensis* (Le et al., 2007).

Effects of cultivation temperature

Cultivation temperature is an important factor affecting *G. lucidum* submerged fermentation (Feng et al., 2016; Yang and Liao, 1998). Effect of temperature on mycelial growth and EPS yield was investigated under the following conditions: inoculum size 0.8 g/flask, rotary speed 160 rpm, and natural initial pH (around 6.0). As depicted in Fig. 2D, cultivation temperature significantly affected mycelial biomass and EPS production. Their production increased with increasing cultivation temperature up to 30 °C, reaching 11.36 g/L biomass and 1.26 g/L EPS, but there was little difference between 28 and 30 °C. Higher or lower temperatures caused a reduction in mycelial growth and EPS production. Thus, a cultivation temperature between 28 and 30 °C was optimal for both mycelial growth and EPS production. This value is slightly lower than that found by Yang and Liao (1998), where the optimal temperature was found to be between 30 and 35 °C for EPS production by *G. lucidum*. In subsequent experiments, temperature was controlled at 28 °C for the submerged cultivation.

Effects of initial pH

It is known that the initial pH of culture medium may affect cell membrane function, cell morphology and structure, and product formation (Shu and Lung, 2004). Initial pH of the medium has been reported to be another key parameter

influencing mycelial growth and EPS production by *G. lucidum* (Fang and Zhong, 2002; Kim et al., 2006b). The effect of initial pH on mycelial growth and EPS production was investigated under the following conditions: inoculum size 0.8 g/flask, rotary speed 160 rpm, and temperature 28 °C. As shown in Fig. 2E, mycelial growth was poor at 6.53 g/L at an initial pH of 3, and abundant growth was observed in pH values above 4.0, with the highest biomass yield (11.36 g/L) observed at initial pH of 5.0. However, in a previous study by Lee et al. (1999), the favorable pH for cell growth of *G. lucidum* was 3.0. EPS production was relatively low at pH values of 3.0 and 4.0, and the highest yield (1.28 g/L) occurred at pH 6.0, with a relatively high biomass (11.13 g/L). Contrary to this result, lowering the initial pH from 6.5 to 3.5 gradually led to a higher EPS production (Fang and Zhong, 2002). In subsequent experiments, as the pH of the medium after sterilization was around 6.0, which favors EPS formation and mycelial growth, the medium was used directly without pH adjustment.

Optimization of EPS production by RSM using BBD design

Predictive model of response

The optimization of culture conditions is crucial for the improvement of microbial fermentation bioprocesses (Gao et al., 2012; Wang et al., 2014). In the present study, as the solid seed was composed of numerous mycelia-covered tiny particles, and inoculation density has a significant effect on *G. lucidum* submerged cultivation, it is reasonable to speculate that rotary speed may significantly affect submerged fermentation performance of the solid seed. Rotary speed generates shear stress in culture broth during shaken cultivation and this influences the dissociation content of the solid seed inoculated. Besides this, moisture content of the solid seed may relate with its dissociation in culture medium due to its close relevance with particle structure and gummy texture (Yang et al., 2014). For these considerations, there may exist complex correlations for EPS production during submerged fermentation among these three variables: moisture content of the solid seed, inoculum size, and rotary speed. Therefore, these three process variables were selected for further optimization. As optimal temperature and initial pH shown in the single factor experiments involving the solid seed were similar to most published studies regarding submerged fermentation of *G. lucidum* for EPS production (Chang et al., 2006; Feng et al., 2016; Kim et al., 2006b; Yang and Liao, 1998), these two variables were not included for further optimization.

The design matrix of the three selected variables and corresponding experimental results are shown in Table 2.

By applying multiple regression analysis on the experimental data, the following second-order polynomial equation was established:

$$Y = 1.29 - 0.057x_1 + 0.12x_2 + 0.031x_3 + 0.042x_1x_2 + 0.047x_1x_3 - 0.16x_2x_3 - 0.24x_1^2 - 0.12x_2^2 - 0.22x_3^2 \quad (3)$$

where Y is the EPS production, x_1 is the coded value of the moisture content of the solid seed, x_2 is the coded value of inoculum size, and x_3 is the coded value of the rotary speed.

As shown in Table 3, the “Model F -value” of 23.58 with p value of 0.0002 indicated that the model was highly significant. The “Lack of Fit F -value” was 5.19 and the “Lack of Fit p value” was 0.0728, indicating that the Lack of Fit was not significant relative to the pure error. The coefficient of determination R^2 was 96.81%, indicating that 96.81% of the variability in the response could be explained by this model. All these results showed a good agreement between the experimental and predicted values. The linear coefficients x_1 and x_2 , the interaction term coefficient x_2x_3 , and the coefficients of the quadratic term x_1^2 , x_2^2 , and x_3^2 were significant, as their p values were below 0.05. According to the p value of the coefficient of x_2x_3 , it can be concluded that the interaction of inoculum size and rotary speed had a significant effect on EPS yield.

Response surfaces and contour plots showing the effect of the three independent variables on EPS production

The response surfaces and contour plots of EPS production are shown in Fig. 3, which depict the interactions between

the two variables by keeping other variables at their zero levels. Figure 3A shows that EPS production increased with increase in the moisture content of solid seed up to approximately 70%, and then decreased. EPS production increased with increasing inoculum size up to near 0.9 g/L, and then began to slightly decrease. Figure 3B shows that EPS production increased with increases of rotary speed and the moisture content of solid seed up to their respective around middle levels, and thereafter it continued to decline. Figure 3C shows that EPS production increased rapidly as rotary speed increased up to around 175 rpm, but beyond this point, it slightly decreased. The inoculum size had similar effects on EPS production as that of rotary speed. The maximal EPS production of 1.29 g/L was predicted by means of Design-Expert software at conditions coded 0.05, 1.00, and -0.07 for the moisture content of solid seed, inoculum size, and rotary speed, respectively. For actual consideration, the best conditions were a 70% moisture content of solid seed, a rotary speed of 160 rpm, and an inoculum size of 0.8 g/flasks for the submerged cultivation of *G. lucidum* for EPS production.

Verification of the model

To validate the adequacy of the model and optimized culture conditions, confirmation experiments were carried out under the obtained optimal conditions (70% moisture content of the solid seed, rotary speed of 160 rpm, and inoculum size of 0.8 g/flask), by holding the temperature at 28 °C, with natural initial pH (around 6.0). The EPS was obtained at 1.33 g/L, which agreed well with the predicted

Table 3 ANOVA analysis for response surface quadratic model for the optimization of EPS production by *G. lucidum*

Source	Statistical analysis				
	Sum of squares	df	Mean square	F -value	Prob > F
Model	0.81	9	0.090	23.58	0.0002
x_1	0.026	1	0.026	6.91	0.0340
x_2	0.11	1	0.11	29.46	0.0010
x_3	7.813E-003	1	7.813E-003	2.04	0.1963
x_1x_2	7.225E-003	1	7.225E-003	1.89	0.2119
x_1x_3	9.025E-003	1	9.025E-003	2.36	0.1686
x_2x_3	0.096	1	0.096	25.10	0.0015
x_1^2	0.24	1	0.24	62.81	< 0.0001
x_2^2	0.062	1	0.062	16.23	0.0051
x_3^2	0.20	1	0.20	51.54	0.0002
Residual	0.027	7	3.829E-003		
Lack of fit	0.021	3	7.108E-003	5.19	0.0728
Pure error	5.480E-003	4	1.370E-003		
Cor total	0.84	16			
$R^2 = 0.9681$					
Adj $R^2 = 0.9270$					
CV = 6.09%

Fig. 3 Response surface (A, B, C) and contour (a, b, c) plots showing the effects of the three independent variables and their interactions on EPS production by *G. lucidum*

value of 1.29 g/L. The model is reliable for predicting EPS production of *G. lucidum* submerged cultivation involving the solid seed.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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