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Enhanced polysaccharide production in mycelium of *Ganoderma atrum* by solid-state fermentation

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Abstract

Polysaccharides are some of the most important bioactive compounds produced by species of *Ganoderma.* In this study, effects of ratio of material to water, the liquid supplement medium including carbon sources, nitrogen sources, and inorganic salts were also studied for the polysaccharide production in mycelium of *Ganoderma atrum* by solid-state fermentation. The seed of *G. atrum* was inoculated in 300 mL cylindrical glass bottles containing 60 g wheat grain at 28 °C for 20 days. One-factor-at-a time and an orthogonal design method were used to establish the optimal liquid supplement medium for maximum polysaccharide production in mycelium. The results showed that the highest polysaccharide production was achieved with ratio of material to water of 1:3, and the optimal liquid supplement medium contained 15 g/L maltose, 10 g/L peptone, 1 g/L KCl and 1.5 g/L $K_2HPO_4 \cdot 3H_2O$. The maximum polysaccharide yield in mycelium was 1.8 mg/g on 20 days. This method first provides an effective way for obtaining polysaccharides in mycelium by solid-state fermentation in *G. atrum*. The strategies used in this study could be widely practical to other fermentation processes.

Key words – culture condition – mycelium polysaccharide – Orthogonal design – optimization

Introduction

Ganoderma (Polyporales, Basidiomycota) is one of the most important Traditional Chinese Medicinal mushrooms with a long history of use in the Orient (Gao et al. 2004, Zhang et al. 2014, Richter et al. 2015). Pharmaceutically active compounds in *Ganoderma* fruiting bodies and mycelium include adenosine, ganoderic acid, lectins, peptides, proteins, polysaccharides, terpenoids and sterols (Li & Wang 2006, Zong et al. 2012, Nie et al. 2013, Zhang et al. 2014, Hapuarachchi et al. 2015). Polysaccharides and triterpenoids are the major source of biological activity and therapeutic (Liu & Zhang 2007). *Ganoderma* has been used as a medicinal agent responsible for immunological regulation (Chen et al. 2014, Shi et al. 2014) and anti-cancer/antitumor (Li & Wang 2006, Zhao et al. 2010, Zhang et al. 2013, Yu et al. 2014, Hapuarachchi et al. 2016), antioxidant (Chen et al. 2008), anti-fungus (Li et al. 2012), anti-hepatitis (Li & Wang 2006), and anti-hyperlipidemia (Meng et al. 2011, Nie et al. 2013).

Submerged culture, solid-state fermentation (SSF) and stationary liquid fermentations are currently used for producing polysaccharides and triterpenoids from *Ganoderma* (Tang & Zhong 2002b, Lee et al. 2003, Hsieh & Yang 2004). Submerged fermentation was considered as a good

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method to increase mycelium, valuable metabolite, polysaccharide, and triterpenoid production (Liu & Zhang 2007). In previous studies, ethyl acetate extracts from *Eupolyphaga sinensis* and *Catharsius molossus*, methanol, ethanol, 1-propanol, 2-propanol, whey permeate and corn oil, were added to the medium for improved polysaccharide production by *G. lucidum* (Liu & Zhang 2007, Song et al. 2007, Huang et al. 2009).

In solid-state fermentations, it was considered that several months were needed to cultivate the *Ganoderma* fruiting bodies and that yield of biomass would be low (Douanla-Meli & Langer 2009). Sources for solid substrate fermentations, such as grain, sawdust or wood, straw, and tea waste are abundant (Yang & Liau 1998, Peksen & Yakupoglu 2009), SSF has therefore become an attractive alternative to submerged culture fermentation for specific applications due to recent improvements in reactor designs (Couto & Sanromán 2006).

In this study, we optimized fermentation medium nutrients to improve polysaccharide production in mycelium of *G. atrum* for the first time. This is one of the most important species of *Ganoderma* used for polysaccharide production. We used a one-factor-at-a time approach and an orthogonal design to improve yields. The effects of ratio of material to water, carbon sources, nitrogen sources, and inorganic salts were studied by solid-state fermentation in 300 mL cylindrical glass bottles.

Materials & Methods

Microorganism

The strain of *G. atrum* was obtained from the culture collection of Huazhong Agricultural University (Hubei, China). The stock culture was maintained on potato dextrose agar (PDA) slants. The slants were inoculated with mycelia and incubated at 28 °C for 10 days.

Seed culture on wheat by solid-state culture

The seed medium was prepared by mixing 60 g of wheat (It was soaked in distilled water for 12h, and was drained for water on wheat grain surface) and 20 mL of distilled water in a 300 mL cylindrical glass bottle (inner diameter 7 mm, height 12 mm) and the bottle was sealed by film of polypropylene plastic. The medium was autoclaved for 30 min at 121 °C. The medium was cooled to room temperature and inoculated with three discs (1 cm^2) from the PDA slants and incubated at 28 °C for 10 days in the dark.

Solid-state fermentation for mycelium

The fermentation medium was prepared by mixing 60 g of wheat (It was soaked in distilled water for 12h, and was drained for water on wheat grain surface) and the liquid supplement medium (carbon sources, nitrogen sources and inorganic salts) in a 300 mL cylindrical glass bottle (inner diameter 7 mm, height 12 mm) and the bottle was sealed by film of polypropylene plastic. The medium was autoclaved for 30 min at 121 °C. The medium was cooled to room temperature and inoculated with 1 spoon wheat grain seed (The biomass weight of mycelium and wheat grain are 10 g) and incubated at 28 °C for 20 days in the dark.

Extraction of polysaccharide in G. atrum mycelium and assay

The experiments were performed in 300 mL cylindrical glass bottles for 20 days. Total fermentation substrates containing 60 g wheat and mycelium of *G. atrum* were mixed with deionized water, and were extracted twice by hot water extraction for one hour each time. All extracts were filtered through gauze of six-layer and finally cooled. The cooled extracts were precipitated with two times volume of 95% (v/v) ethanol overnight in refrigerator at 4 °C. All precipitated samples were centrifuged at 2810 × g for 10 min. The rough polysaccharide in mycelium at the bottom of tubes were collected, and dried to a constant dry weight at 60 °C. Finally, the content of polysaccharide in mycelium was measured using a phenol sulfuric acid method (Douanla-Meli & Langer 2009).

Orthogonal array for medium optimization

In order to evaluate the influence of liquid supplement medium component (maltose, peptone, KCl and $K_2HPO_4 \cdot 3H_2O$) on mycelium polysaccharide production (MPP), L_9 (3^4) orthogonal array was used to evaluate. The L_9 (3^4) orthogonal array was selected to examine the effects of four factors on mycelium polysaccharide production (Table 1). The orthogonal arrays, data analysis and ANOVA were obtained using Design–Expert Version 8.0.5b software package (Stat-Ease Inc., Minneapolis, USA) based on the Taguchi method.

Table 1 Orthogonal experiment factors and levels.

Factors	Symbols	Level 1 (g/L)	Level 2 (g/L)	Level 3 (g/L)
Maltose	A	15	30	45
Peptone	B	10	20	30
KCl	C	0.5	1	1.5
$K_2HPO_4 \cdot 3H_2O$	D	0.5	1	1.5

Statistical analysis

The dry weight of *G. atrum* mycelium polysaccharide (GAMP) production was expressed as means ± SD. An Analysis of Variance (ANOVA) followed by Tukey's test was applied for multiple comparisons of significant analyses at $P < 0.05$. Orthogonal design experiments and statistical data analyses were performed in Statistic Package for Social Science (SPSS) version 17.0 software packet.

Results and discussion

Effects of ratio of material to water for polysaccharide

In order to investigate the effect of ratio of material to water on the polysaccharide production in mycelium, different ratios of material to water (1:1–1:5, m/v) were prepared in this study. The highest polysaccharide production of 1.48 ± 0.10 mg/g was achieved with ratio of material to water of 1:3 (Fig. 1).

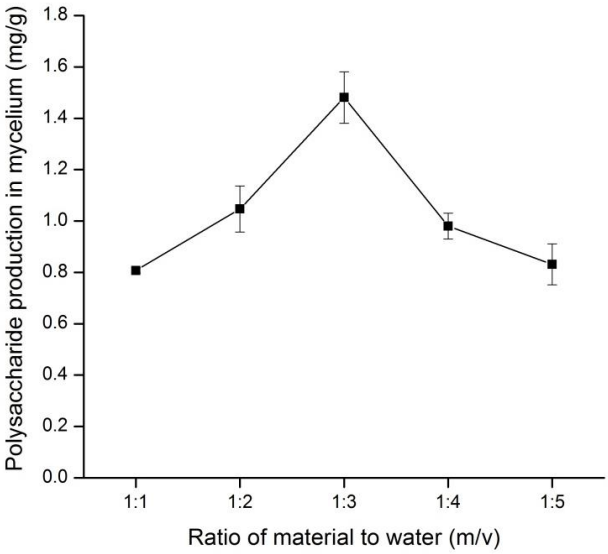

Fig. 1 – Effects of ratio of material to water on the polysaccharide production in *G. atrum* mycelium by solid-state fermentation.

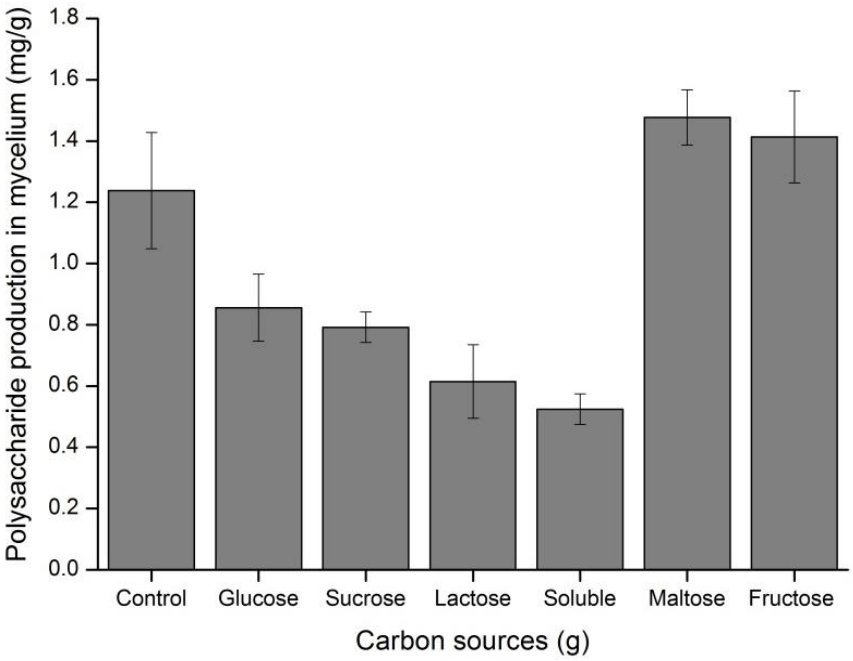

Fig. 2 – Effect of carbon sources on the polysaccharide production in *G. atrum* mycelium by solid-state fermentation.

Effect of carbon sources

To choose a suitable carbon source for polysaccharide in mycelium by *G. atrum*, six carbon sources including glucose, sucrose, lactose, soluble starch, maltose and fructose were prepared at concentrations of 30 g/L in the basal medium. Maltose and fructose were the best carbon sources for polysaccharide production in mycelium (Fig. 2). Polysaccharide production in mycelium reached 1.48 ± 0.09 mg/g with maltose and 1.413 ± 0.15 mg/g with fructose. But there is a difference in this result compare to previous studies (Tang & Zhong 2002a). Possible reasons are imparity of solid substrate or the cultivation method. Maltose was selected as the main carbon source in the remaining experiment.

Effects of nitrogen sources

To find a suitable nitrogen source for polysaccharide production in mycelium, we added various nitrogen sources (soybean meal, peptone, yeast extract, beef extract, NH_4NO_3, NH_4Cl) at a concentration of 20 g/L to the nitrogen-free basal medium (Fig. 3). Peptone and yeast extract dramatically improved the mycelium growth rate of *G. atrum*, and the growth rate was faster when adding peptone as compared to yeast extract. Fermentation substrates of wheat grain were covered with mycelium of *G. atrum* at 15 days. However, growth rate of mycelium was slow when adding NH_4Cl or NH_4NO_3. Organic nitrogen was advantageous to both growth and biosynthesis of metabolites. The result is consistent with the experimental data reported in other medicinal mushroom (Wen et al. 2008, Kang et al. 2012). The results suggested that maximum mycelium polysaccharide production of 1.39 ± 0.08 mg/g was produced with peptone as a nitrogen source.

Inorganic ion is an important nutritional component in medium for mycelium growth (Bae, et al. 2000). In order to investigate the effect of inorganic salt for the polysaccharide production in mycelium by *G. atrum*, various inorganic salts ($MgSO_4 \cdot 7H_2O$, $ZnSO_4$, KH_2PO_4, KCl, $CaSO_4 \cdot 2H_2O$, and $K_2HPO_4 \cdot 3H_2O$) were added to basal medium at a concentration of 1 g/L, individually. All inorganic salts improved polysaccharide production (Fig. 4). The highest polysaccharide production (1.36 ± 0.09 mg/g) was observed in medium, when $K_2HPO_4 \cdot 3H_2O$ was used. At last, $K_2HPO_4 \cdot 3H_2O$ and KCl were recognized as the best two inorganic salts for mycelium polysaccharide production.

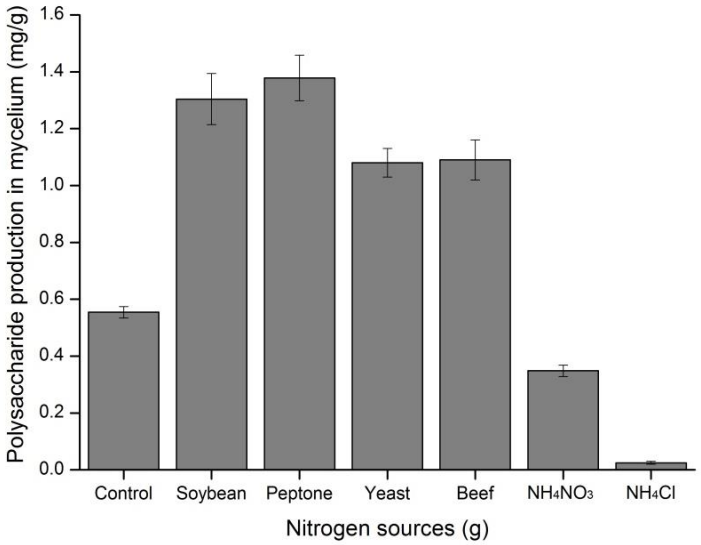

Fig. 3 – Effect of nitrogen sources on the polysaccharide production in *G. atrum* mycelium by solid-state fermentation.

Effects of inorganic salt

Orthogonal experimental design

To investigate the roles of different liquid supplement medium components and optimize their concentrations for polysaccharide production in mycelium, the orthogonal layout L_9 (3^4) method was used. According to the above results achieved using the one-factor-at-a-time method, we selected and separated three levels as shown in Table 1. Table 2 shows the detailed experimental design and results. Nine experiments were carried out in triplicate. The experiment design and ANOVA for the experimental results obtained by SPSS Version software package, and optimal levels of each factor for obtaining higher polysaccharide production in mycelium are given in Table 2 and Table 3, respectively. And Fig. 5 shown the mycelium growth state for polysaccharide of *G. atrum* mycelium at 20 days by L_9 (3^4) orthogonal design.

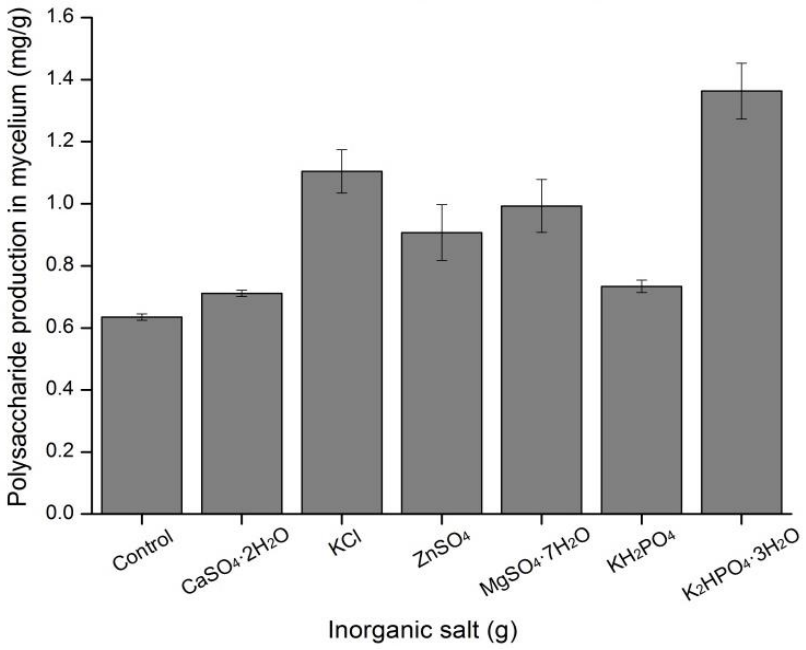

Fig. 4 – Effect of inorganic salt on the polysaccharide production in *G. atrum* mycelium by solid-state fermentation.

Table 2 The L_9 (3^4) orthogonal array applied for the polysaccharide production in *G. atrum* mycelium by solid-state fermentation.

Run	Variables Code				Polysaccharide production（mg/g, n=3）			
	A	*B*	*C*	*D*	Y_1	Y_2	Y_3	$\overline{Y}$
1[a]	1	1	1	1	1.48	1.40	1.41	1.43[b]
2	1	2	2	2	1.08	1.05	1.20	1.11
3	1	3	3	3	0.52	0.55	0.60	0.56
4	2	1	2	3	1.55	1.46	1.60	1.54
5	2	2	3	1	0.90	1.00	0.92	0.94
6	2	3	1	2	0.57	0.58	0.60	0.58
7	3	1	3	2	1.18	1.18	1.42	1.26
8	3	2	1	3	1.12	0.78	0.87	0.92
9	3	3	2	1	0.57	0.47	0.48	0.51
K_1	3.0963[c]	4.2276	2.9367	2.8752				
K_2	3.0588	2.9709	3.1524	2.9532				
K_3	2.688	1.6443	2.754	3.0144				
k_1	1.0321[d]	1.4092	0.9789	0.9584				
k_2	1.0196	0.9903	1.0508	0.9844				
k_3	0.8960	0.5481	0.9180	1.0048				
R	0.1361[e]	0.8611	0.1328	0.0463				
Optimal level	1	1	2	3				

[a]: The arrangements of column *A*–*D* were decided by orthogonal design for L_9 (3^4).
[b]: Average of triple determinations.
[c]: $K_i^A = \sum$ mycelia polysaccharide yield at A_i. Values are mean of triple determinations.
[d]: $k_i^A = K_i^A / 3$. Values are mean of triple determinations.
[e]: $R_i^A = \max\{K_i^A\} - \min\{k_i^A\}$. Values are mean of triple determinations.

Table 3 The ANOVA of the results of L_9 (3^4) orthogonal design for the polysaccharide production in *G. atrum* mycelium.

Source	Sum of quares	df	Mean Square	*F-value*	*p*-value Prob > *F*
A	0.102	2	0.051	6.40	0.008**
B	3.338	2	1.669	209.73	0.000**
C	0.080	2	0.040	5.00	0.019**
D	0.010	2	0.005	0.61	0.554
Error	0.143	18	0.008		
Total	3.672	26			

Pred-R^2 = 0.961; Adj-R^2 = 0.944; ** 1% significance level.

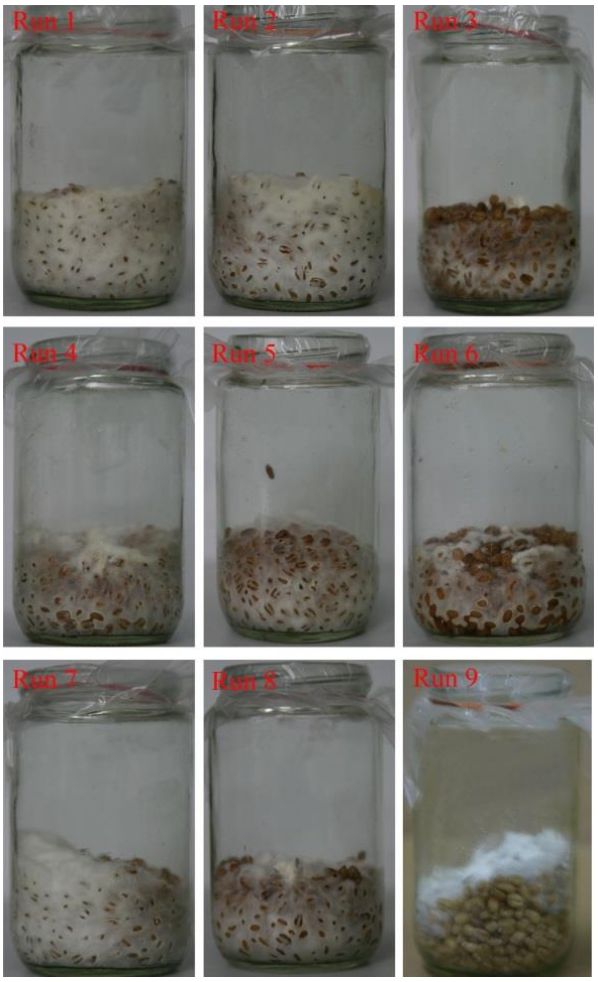

Fig. 5 – The mycelium growth state for polysaccharide of *G. atrum* mycelium at 20 days by L_9 (3^4) orthogonal design.

As shown in Table 2, it is evident that maximum polysaccharide production of mycelium by *G. atrum* of 1.54 mg/g could be achieved using a combination of coded levels (*A*, maltose, level 1; *B*, peptone, level 1; *C*, KCl, level 2; *D*, $K_2HPO_4 \cdot 3H_2O$, level 3), and actual values for various factors were 15 g/L maltose, 10 g/L peptone, 1 g/L KCl, 1.5 g/L $K_2HPO_4 \cdot 3H_2O$, respectively.

According to the magnitude order of R (Max Dif), the order of effect of all factors on polysaccharide production could be determined, and was peptone > maltose > KCl > $K_2HPO_4 \cdot 3H_2O$. Analysis of ANOVA (Table 3) showed that peptone, maltose, and KCl should be effective ingredients for improved polysaccharide production ($P < 0.05$). Nevertheless, $K_2HPO_4 \cdot 3H_2O$ was not significant ($P > 0.05$). Peptone was more significant than all other ingredients.

Conclusions

In this work, the one-factor-at-a time method and orthogonal design method were employed to establish the key factors and identify optimal culture conditions to improve the polysaccharide production in *G.* atrum mycelium by solid-state fermentation for the first time. The results showed that the highest polysaccharide production was achieved with ratio of material to water of 1:3, and the optimal liquid supplement medium contained 15 g/L maltose, 10 g/L peptone, 1 g/L KCl and 1.5 g/L $K_2HPO_4 \cdot 3H_2O$. The maximum polysaccharide yield in mycelium was 1.8 mg/g on 20 days. This method first provides an effective way for increasing polysaccharides production by solid-state fermentation in *G. atrum.* The strategies used in this study could be widely practical to other fermentation processes.

Acknowledgements

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