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Selection of a Culture Medium for Reducing Costs and Enhancing Biomass and Intracellular Polysaccharide Production by *Agaricus blazei* AB2003

Gao-Qiang Liu and Xiao-Ling Wang*

College of Life Sciences and Technology, Central South University of Forestry and Technology, 498 South Shaoshan Road, CN-410004 Changsha, PR China

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Summary

A practical medium for reducing costs and enhancing both biomass and intracellular polysaccharide (IPS) production by a newly screened *Agaricus blazei* AB2003 has been selected. The results show that IPS production was growth-associated, that a combination of corn flour extract and glucose was the best carbon source, and that a combination of wheat bran extract and yeast extract was the best nitrogen source for both biomass and IPS production. The effects of the four factors were further investigated using four-factor, three-level orthogonal test design. The best combination for the production of biomass and IPS was (in g/L): corn flour extract 15, glucose 6, yeast extract 3, and wheat bran extract 6. The maximum biomass and IPS in a 30-litre stirred tank bioreactor reached 11.30 and 0.72 g/L in the optimized medium, respectively. The use of two low-cost raw materials like corn flour and wheat bran extract as the major medium constituents could reduce the costs of biomass and IPS production.

Key words: *Agaricus blazei*, intracellular polysaccharide, mycelial growth, medium selection, submerged fermentation

Introduction

Mushrooms have recently become attractive as a functional food (*1*,*2*). Studies have been focused on the polysaccharide production by mushrooms in submerged cultures (*3*,*4*). *Agaricus blazei* Murrill is an edible and medicinal mushroom which is popularly consumed due to its antitumour properties (*5*). The fungus is particularly rich in polysaccharides, and has demonstrated effective results in treating and preventing cancer (*6–8*). Recently, it has been used for the prevention of cancer and/or as an adjuvant with cancer chemotherapy drugs after the removal of a malignant tumour (*9*). Moreover, the fungus has been shown to be effective in treating AIDS, diabetes, hypertension, and hepatitis (*10*).

Because it usually takes several months to cultivate the fruiting body of *A. blazei* and it is also difficult to control the product quality during its cultivation, there is a great need to regularly supply the market with enough high-quality *A. blazei* products. Submerged fermentation of *A. blazei* is viewed as a promising alternative for production of mycelial biomass and polysaccharides (*11*,*12*). In our previous work, the production of biomass and extracellular polysaccharides (EPS) by *A. blazei* AB002 was studied (*13*). Nevertheless, data on enhancing both biomass and intracellular polysaccharide (IPS) production from *A. blazei* are scarce, and there is little attention given to the production of biomass and polysaccharides by *A. blazei* using a low-cost culture medium.

The objective of this study is to select a cheap culture medium for enhanced production of both biomass and IPS by a newly screened strain, *A. blazei* AB2003.

*Corresponding author; Phone: ++86 731 5623 392; Fax: ++86 731 5623 498; E-mail: gaoliuedu@yahoo.com.cn

Materials and Methods

Microorganism and culture conditions

The strain of *A. blazei* AB2003 was screened and collected by Strain Collection of Industrial Microorganisms (SCIM), Central South University of Forestry and Technology, PR China.

Preculture medium consisted of the following components (in g/L): glucose 20, peptone 4, $KH_2PO_4 \cdot H_2O$ 0.5, and $MgSO_4 \cdot 7H_2O$ 0.3. For the preculture, a 60-mL medium was prepared in a 250-mL flask, 5 mycelial agar discs (0.5 cm) were inoculated, and followed by 8 days of incubation at 26 °C on a rotary shaker (160 rpm).

For the submerged fermentation, 100 mL of the medium were prepared in a 500-mL flask, and preculture broth was inoculated at 10 % (by volume). All fermentation experiments were carried out at 26 °C on a rotary shaker at 160 rpm for 7 days unless otherwise specified in the Results section.

The conditions for fermentation in a 30-litre stirred tank bioreactor were as follows: working volume 20 L, stirring rate 160 rpm, aeration rate 1.5 vvm, culture temperature 26 °C, and culture time 9 days.

Substrate materials

Glucose, fructose, sucrose, maltose, starch, NH_4NO_3, NH_4Cl and KNO_3 were purchased from Sinopharm Chemical Reagent Co., Ltd, PR China, while corn flour, rice bran, wheat bran and bean powder were purchased from Hongxing Agricultural Product Co., Ltd., PR China.

Effect of carbon source

The effect of carbon source on the fungal culture was investigated by using various sources, *e.g.* glucose, fructose, sucrose, maltose, starch, corn flour extract and rice bran extract (20 g/L); and glucose with corn flour extract, sucrose with corn flour extract, glucose with rice bran extract and sucrose with rice bran extract (10 g/L of each component). For observation, each carbon source was added to the cultures as the sole source of carbon.

Effect of nitrogen source

Organic nitrogen sources included yeast extract, peptone, wheat bran extract (4 g/L); and peptone with yeast extract, peptone with wheat bran extract, yeast extract with wheat bran extract, peptone with bean powder extract and yeast extract with bean powder extract (2 g/L of each component), while inorganic nitrogen sources included NH_4NO_3, NH_4Cl and KNO_3 (4 g/L). Each nitrogen source was added to the cultures as the sole source of nitrogen.

Preparation of extracts from corn flour, rice bran, wheat bran and bean powder

A mass of 1 kg of oven dried and milled corn flour, rice bran, wheat bran or bean powder was suspended in a solution containing 10 L of distilled water and 50 mL of H_2SO_4 and then autoclaved (1 atm) for 20 min. The liquid fraction was separated by filtration, pH was adjusted to 6.5 using NaOH solution and the extract was obtained by the removal of water at 50 °C under reduced pressure. Total sugar, reducing sugar and gross protein of each extract were assayed.

Determination of mycelial biomass and IPS

Samples collected at various intervals from shake flasks and bioreactor were filtered using a 40-mesh stainless sieve and the mycelium was harvested. Biomass was obtained by centrifuging the mycelium at 10 000 rpm for 15 min, washing the precipitated cells three times with distilled water, and drying at 60 °C for sufficient time to a constant mass (*1–2*).

For the analysis of IPS, the dried mycelia were extracted with 1 M NaOH at 60 °C for 1 h, and then the supernatant was assayed by phenol sulphuric acid method (*2*).

Measurement of reducing sugar, total sugar and gross protein

Residual sugar in the broth and reducing sugar in each extract were assayed by 3,5-dinitrosalicylic acid method (*14*). Total sugar concentration in each extract was determined by phenol sulphuric acid method (*2*). Gross protein content in the broth and each extract was determined by biuret reaction (*15*).

Orthogonal matrix design

According to the results of one-factor-at-a-time experiments, the 3^k factorial design, a factorial arrangement with *k* factors each at three levels, was employed (Table 1). Detailed experimental conditions for each project are listed in Table 2.

Table 1. Experimental factors and their levels for the orthogonal design

Level	A/(g/L)	B/(g/L)	C/(g/L)	D/(g/L)
1	6	6	3	3
2	10	10	4	4
3	15	15	6	6

Letters A, B, C and D represent factors of corn flour extract, glucose, yeast extract and wheat bran extract, respectively
Numbers 1, 2 and 3 represent concentration levels of each factor

Statistical analysis

The analyses were made at least in triplicate replication and results presented were expressed as mean±S.D. Statistical analysis was performed using one way analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT). The p-values$\leq$0.05 were considered significant and p$\leq$0.01 were extremely significant.

Results

The biochemical composition of the extracts from corn flour, rice bran, wheat bran and bean powder is given in Table 3. Both corn flour and rice bran extracts can be used as carbon sources because their total sugar mass fractions were 78.2 and 67.8 %, respectively, while the wheat bran and bean powder extracts can be used as nitrogen sources due to their high mass fractions of gross protein.

Table 2. Application of L_9 (3^4) orthogonal design for evaluation of mycelial growth and IPS production by *A. blazei*

Run	A	B	C	D	γ(biomass)/(g/L)	γ(IPS)/(g/L)
1	1	3	1	2	5.1±0.31	0.36±0.05
2	1	1	2	1	5.7±0.23	0.41±0.04
3	1	2	3	3	3.1±0.27	0.31±0.03
4	2	2	1	1	7.0±0.39	0.57±0.06
5	2	3	2	3	5.7±0.36	0.42±0.06
6	2	1	3	2	5.0±0.27	0.42±0.05
7	3	1	1	3	9.1±0.41	0.63±0.11
8	3	2	2	2	6.7±0.27	0.46±0.05
9	3	3	3	1	4.7±0.23	0.34±0.04

The arrangements of columns A, B, C and D were designed by orthogonal design for 4 (factor)×9 (run number), each row of the run number represents one experiment replicate. Each run was carried out three times. Values are mean±SD of three determinations. The experiment was carried out at 26 °C on a rotary shaker at 160 rpm for 7 days

Table 3. Biochemical constituents of the extracts from corn flour, rice bran, wheat bran and bean powder

Extracts	Fraction of constituents/%		
	Reducing sugar	Total sugar	Gross protein
Corn flour	43.7±3.61	78.2±4.29	7.1±0.61
Rice bran	39.2±1.80	67.8±4.75	12.6±1.11
Wheat bran	35.6±1.62	58.3±3.45	22.2±1.82
Bean powder	24.3±2.34	50.8±1.03	29.3±2.03

Effect of carbon source

Effects of different carbon sources on the cell growth of *A. blazei* were investigated. Analysis of variance showed that there was an extremely significant difference in both biomass and IPS concentration, *i.e.* 4.11–6.73 and 0.26–0.49 g/L, respectively, $p \leq 0.01$, with different carbon sources (Table 4), and it is obvious that IPS production was growth-associated. The most suitable medium for production of both biomass and IPS was a combination of corn flour extract and glucose, when the highest biomass and IPS concentrations were 6.73 and 0.49 g/L, respectively. These results suggest that a combination of corn flour extract and glucose had complementary effects in promoting the cell growth and IPS production. It is reasonable that a combination of corn flour extract and glucose was chosen as the carbon source in subsequent experiments. Similar results that a combination of corn flour and glucose contributed to the highest yield of the mycelia by *Hericium erinaceus* were also reported (*16*).

Effect of nitrogen source

Table 5 shows the effects of nitrogen source on mycelial growth and IPS production. It was found that there were extremely significant changes in biomass and IPS concentrations, *i.e.* 3.42–7.17 and 0.14–0.52 g/L, respectively, $p \leq 0.01$, when various nitrogen sources were used.

Table 4. Effect of carbon source on mycelial growth and IPS production by *A. blazei*

Carbon source	γ(biomass)/(g/L)	γ(IPS)/(g/L)
Glucose	5.93±0.81	0.40±0.10
Fructose	5.41±0.61	0.21±0.08
Sucrose	5.82±0.71	0.38±0.11
Maltose	5.81±0.62	0.36±0.09
Starch	5.11±0.43	0.37±0.06
Corn flour	5.02±0.41	0.32±0.04
Rice bran	3.09±0.40	0.21±0.05
Glucose+corn flour extract*	6.73±0.81	0.50±0.10
Sucrose+corn flour extract*	6.51±0.47	0.43±0.07
Glucose+rice bran extract*	4.63±0.21	0.39±0.11
Sucrose+rice bran extract*	4.21±0.56	0.33±0.05

*Used at 10 g/L, other carbon sources were used at 20 g/L
The experiment was carried out at 26 °C on a rotary shaker at 160 rpm for 7 days

Table 5. Effect of nitrogen source on mycelial growth and IPS production by *A. blazei*

Nitrogen source	γ(biomass)/(g/L)	γ(IPS)/(g/L)
Peptone	5.63±0.61	0.31±0.06
Yeast extract	6.83±0.49	0.43±0.05
Wheat bran	5.72±0.17	0.32±0.11
Peptone+yeast extract*	6.85±0.59	0.49±0.09
Peptone+wheat bran*	6.73±0.78	0.41±0.07
Yeast extract+wheat bran*	7.17±0.67	0.52±0.11
Peptone+bean powder*	4.71±0.45	0.28±0.06
Yeast extract+bean powder*	5.71±0.64	0.33±0.07
NH_4NO_3	3.61±0.23	0.17±0.03
NH_4Cl	3.42±0.26	0.16±0.02
KNO_3	3.51±0.31	0.14±0.03

*Used at 2 g/L, other carbon sources were used at 4 g/L
The experiment was carried out at 26 °C on a rotary shaker at 160 rpm for 7 days

Compared with inorganic nitrogen sources, organic nitrogen sources supported both mycelial growth and IPS production. This result is in agreement with the data reported in literature (*11*). Kim *et al*. (*12*) reported that a combination of yeast extract and soytone peptone was a suitable nitrogen source for mycelium and EPS production by *A. blazei*. In this study, the maximum effect on both cell growth and IPS production was observed with a combination of yeast and wheat bran extracts.

Optimization results by the orthogonal matrix method

The experimental data about biomass and IPS production using orthogonal design are shown in the last two columns in Table 2. Analyses of the influence of media on biomass and IPS production are shown in Table 6. The results show that the effects of four key medium

Table 6. Analysis of the influence of media on biomass and IPS production in shake flask culture of *A. blazei* with orthogonal design

	γ(biomass)/(g/L)				γ(IPS)/(g/L)			
	A	B	C	D	A	B	C	D
K1	14.0±0.37	19.8±0.42	21.2±0.41	17.4±0.48	1.08±0.09	1.46±0.12	1.56±0.11	1.32±0.09
K2	17.7±0.81	16.8±0.84	18.1±1.09	16.8±0.85	1.41±0.12	1.34±0.15	1.19±0.08	1.25±0.12
K3	20.5±0.33	15.5±0.12	12.8±0.59	17.9±1.14	1.43±0.05	1.12±0.06	1.07±0.12	1.36±0.07
*k*1	4.7±0.07	(6.6±0.04)*	(7.1±0.13)*	5.8±0.16	0.36±0.01	(049±0.01)*	(0.52±0.01)*	0.44±0.03
*k*2	5.9±0.27	5.6±0.28	6.0±0.36	5.6±0.28	0.47 ± 0.04	0.46±005	0.40±0.03	0.42±0.04
*k*3	(6.8±0.06)*	5.2±0.02	4.3±0.02	6.0±0.38	(0.48±0.01)*	0.37±0.02	0.36±0.02	0.45±0.03
R	2.1±0.43	1.4±0.32	2.8±0.33	0.4±0.64	0.12±0.36	0.12±0.08	0.16±0.08	0.03±0.07
Optimal level	1	2	3	3	1	2	3	3

*The value using this concentration is significantly higher than that using other concentrations ($p<0.05$)

variables on biomass production were consistent with their effects on IPS production. There were significant differences in the tested concentrations of corn flour extract, glucose, and yeast extract ($p\leq0.05$); however, no significant difference was observed in the tested concentrations of wheat bran extract ($p>0.05$). According to the range, the order of the effect of all factors on mycelial growth and IPS production was yeast extract, followed by corn flour extract, glucose and wheat bran extract in sequence, and the optimal levels were 1 (3 g/L), 3 (15 g/L), 1 (6 g/L) and 3 (6 g/L), respectively. The levels were the same as those in run 7 (three repeats, Table 2). Therefore, it is not necessary to verify the optimization results obtained by the orthogonal matrix method.

Based on the above results, the optimal medium for both biomass and IPS production was (in g/L): corn flour extract 15, glucose 6, yeast extract 3, wheat bran extract 6, KH_2PO_4 0.5 and $MgSO_4{\cdot}7H_2O$ 0.3.

Fermentation curves in shake flask and a 30-litre stirred tank bioreactor

To observe the substrate consumption, and biomass and IPS production through the whole fermentation process with the optimized medium, the time courses of mycelial growth, IPS production and substrate consumption in shake flask are shown in Fig. 1a, while Fig. 1b shows the time profiles of mycelial growth, IPS production and substrate consumption in a 30-litre stirred tank bioreactor with the optimized medium. It is shown that the concentrations of residual sugar in shake flask and stirred bioreactor sharply decreased to 2.0 g/L on the 7th day and to 1.6 g/L on the 6th day, respectively. Both biomass and IPS production were further enhanced in the bioreactor with the optimized medium, and 11.30 g/L of biomass and 0.72 g/L of IPS were obtained (Fig. 1b), suggesting that the medium selected for the study is also suitable for both biomass and IPS production at large scale.

Discussion

Recently, submerged fermentation of *A. blazei* has been viewed as a promising alternative for production of mycelial biomass and polysaccharides (*11–13*). However, the growth of mycelium of *A. blazei* was very slow and the yield of mycelial biomass was much lower than that of other macrofungi, such as *Ganoderma lucidum* (*2*), *Lentinula edodes* (*17*), and *Tremella aurantialba* (*18*). Therefore, to reduce the production cost of *A. blazei* at large scale, the use of some potential low-cost substrates is highly necessary.

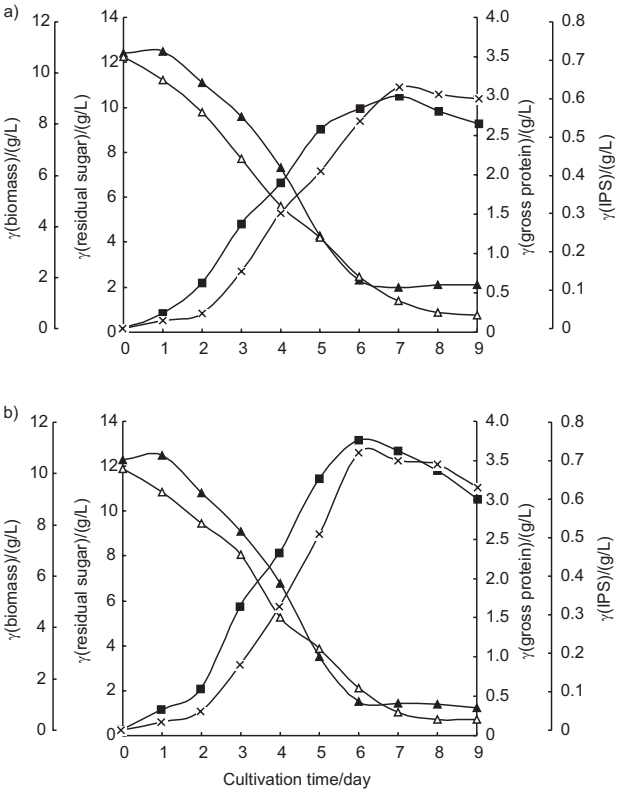

Fig. 1. Time courses of mycelial growth (■), IPS production (×), sugar consumption (▲) and protein consumption (□) by *A. blazei* grown in (a) shake flask and (b) 30-litre stirred tank bioreactor with the optimized culture medium (in g/L): corn flour extract 15, glucose 6, yeast extract 3, wheat bran extract 6, KH_2PO_4 0.5, $MgSO_4{\cdot}7H_2O$ 0.3. Fermentation in the shake flask was carried out at 26 °C on a rotary shaker at 160 rpm for 9 days. Bioreactor conditions: working volume 20 L, stirring rate 160 rpm, aeration rate 1.5 vvm, culture temperature 26 °C and culture time 9 days

In the present study, a practical medium for reducing costs and enhancing both biomass and IPS production by *A. blazei* AB2003 in submerged fermentation was selected. The results suggest that a combination of glucose, yeast extract, corn flour extract and wheat bran extract had complementary effects in promoting both biomass and IPS production, and the best combination for the production of biomass and IPS was (in g/L): corn flour extract 15, glucose 6, yeast extract 3, and wheat bran extract 6, when the maximum biomass and IPS in a 30-litre stirred tank bioreactor reached 11.30 and 0.72 g/L, respectively. It is notable that the use of corn flour and wheat bran extract as the major media could reduce the costs of the production. Nowadays, the price of wheat bran from industry is only about 4 % of that of yeast extract, and 5 % of peptone, and the price of corn flour is only about 25 % of glucose.

In a previous work (*11*), 50 g/L of malt extract were cited as a suitable carbon source for mycelium and IPS production by *A. blazei* under optimal culture conditions, when 10.83 g/L of biomass and 0.25 g/L IPS were obtained. Another study reported that a combination of glucose and dextrin was the most suitable carbon source, and a combination of yeast extract and soytone peptone was the suitable nitrogen source for mycelium and exopolysaccharide production by *A. blazei* (*12*). With the optimized medium composition, the highest mass concentration of the biomass was 9.85 g/L.

Conclusion

The medium selected in this work was suitable for the production of both biomass and IPS by *A. blazei* AB2003, and the shake flask process was successfully reproduced in a 30-litre stirred tank bioreactor. The maximum biomass and IPS in the stirred tank bioreactor reached 11.30 and 0.72 g/L in the optimized medium, respectively. The use of two low-cost raw materials of corn flour and wheat bran extract as the major medium constituents could reduce the costs of biomass and IPS production.

In conclusion, this work provided a low-cost culture medium for enhanced production of both biomass and IPS by a newly screened strain, *A. blazei* AB2003, at large scale.

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