

Application of Statistical Analysis for the Optimization of Mycelia and Polysaccharide Production by *Tremella aurantialba*

Zhicai Zhang^{1,2}, Yin Li^{1,3} and Kechang Zhang^{1,2*}

¹Key Laboratory of Industrial Biotechnology, Ministry of Education, Southern Yangtze University, Wuxi 214036, PR China

²Lab of Biomass Resources, School of Biotechnology, Southern Yangtze University, Wuxi 214036, PR China

³Department of Plant Science, North Dakota, State University, Fargo ND 58105, USA

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Summary

Statistical analyses were applied to optimize the medium composition for the mycelial growth and polysaccharide production by *Tremella aurantialba* in shake flask cultures. Firstly, four significant factors (xylan, peptone, wheat bran and corn powder) on mycelial growth and polysaccharide yield ($p \leq 0.05$) were obtained using one-at-a-time design. Subsequently, in order to study the mutual interactions between variables, the effects of these factors were further investigated using four-factor, three-level orthogonal test design and the optimal composition was (in g/L): xylan 40, peptone 10, wheat bran 20, corn powder 20, KH_2PO_4 1.2 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.6. Finally, the maximum mycelium yield and polysaccharide production in 50-litre stirred-tank bioreactor reached 36.8 and 3.01 g/L under the optimized medium, respectively.

Key words: *Tremella aurantialba*, optimization, orthogonal test, polysaccharide, statistical analysis

Introduction

Tremella aurantialba, extensively used in Chinese medicine, is a wood-inhabiting host-specific macrofungus. During the last 15–20 years, scientific studies on *T. aurantialba* polysaccharides from fruit body and mycelium, and the crude extraction of the broth have been carried out in China and Japan. It has been reported that the composite of *T. aurantialba* decoction remarkably relaxes the tracheal smooth muscle and is anti-asthmatic (1). The report also shows that oral administration or subcutaneous injection of crude extract of *T. aurantialba* to mice can raise macrophages, enhance phagocytic function, adjust the effects on specific immunocompetence of mice, and increase mice nonspecific immunocompetence (2). The

fermented products from *T. aurantialba* can remarkably prolong the clotting time of the thrombogen and increase the volume of the blood flow in the meninges (3). The polysaccharides from fruit bodies of *T. aurantialba* exhibited remarkable hypoglycemic activity in normal mice, streptozotocin-induced diabetic mice and genetically diabetic mice following intraperitoneal administration (4). The mycelium polysaccharides produced by *T. aurantialba* were reported to prevent and cure diet-induced hyperlipidemia, relieving cough and reducing phlegm, curing cardiovascular and cerebral diseases, enhancing haemopoietic function, and increasing body's immunity (5–7). Zhang *et al.* (8) reported that the polysaccharides from the mycelia could decrease the blood sugar content. Therefore, it would be favourable to in-

*Corresponding author; Phone/Fax: ++86 510 85 809 237; E-mail: zhangzhicai2001@yahoo.com.cn

crease the yield of mycelia in fermentation of *T. aurantialba* and to improve the content of polysaccharides.

Orthogonal design is one of the important statistical methods that use the Taguchi parameter design methodology (9). It is feasible to investigate the influence of controlled factors in a multivariable system using this method. It can also give effective responses in the course of system optimization. This method has been successfully applied for the optimization of culture media to improve mycelial growth and exopolysaccharide production in the fermentation process (10–12). The objective of this study is to optimize submerged culture for the production of mycelial biomass and endopolysaccharide in *T. aurantialba* using a statistical experimental design. In the first step, a mono-factor was used to determine the effects of four possible medium variables on mycelial growth and endopolysaccharide production, and then the concentrations of the medium composition that had significant effect on the growth and production were further optimized using orthogonal design.

Materials and Methods

Microorganism and media

Tremella aurantialba was kindly provided by Prof. Weijing Qu, East China Normal University. The stock culture was maintained on potato glucose agar (PGA) slant. The slants were incubated at 28 °C for 7 days and then stored at 4 °C. The fungus was grown in 35 g of solid seed medium containing 15 g of wheat bran and 20 mL of water. Solid seed was incubated at 25 °C for 7 days. The liquid seed culture was grown in a 250-mL flask containing 60 mL of medium, which contained (in g/L): xylan 20 (Kangyuan Biotechnology plant, Jiangsu), corn powder 10, and peptone 5, incubated in a rotary shaker at 150 rpm and 27 °C for 2 days.

Submerged incubation and fermentation

T. aurantialba was initially grown on PGA medium in a test tube, and then transferred into the solid seed medium. During the culturing period, the flasks were shaken once per day from the third to the fifth day. The solid culture (5 g or so) was transferred into the liquid seed medium. Unless otherwise specified, fermentation was carried out in a flask containing 60 mL of the medium at 27 °C for 7 days.

Bioreactor fermentation

The fermentation medium was inoculated with 10 % (volume ratio) of the liquid seed culture and then cultivated in a 50-litre stirred-tank fermentor (made in Jiangsu University, China). Unless otherwise specified, fermentations were performed under the following conditions: agitation speed 150 rpm, initial pH=6.0, working volume 30 L. The reactor was aerated by a ring sparger and stirred by a four-blade Rushton turbine impeller.

Orthogonal matrix method

The 3^k factorial design, a factorial arrangement with k factors at all three levels, was employed. Factors and interactions were denoted by capital letters. The three levels of the factors were set as low, intermediate and

high. Each treatment combination in the 3^k designs was denoted by k digits, where the first digit indicated the level of the factor A, the second digit indicated the level of the factor B, and the kth digit indicated the level of the factor k. For a problem with four design variables and three levels, the minimum orthogonal matrix method was selected as L₉ (3⁴), as noted in Table 1. A logical next step was to determine the point in the important factors that led to the best possible response (10,13). Detailed experimental conditions for each project are listed in Table 2.

Table 1. Experiment factors and their levels for the orthogonal projects

Level	A/%	B/%	C/%	D/%
1	4	0.5	1.0	1.0
2	6	1.0	1.5	1.5
3	8	1.5	2.0	2.0

Symbols A, B, C and D represent factors of xylan, peptone, wheat bran and corn powder, respectively. Symbols 1, 2 and 3 represent concentration levels of each factor

Table 2. Application of L₉ (3⁴) orthogonal projects to mycelial growth and polysaccharide projections by *T. aurantialba*

Run	A	B	C	D	γ (dry mycelia) g/L	γ (mycelial polysaccharides) g/L
1	1	3	1	2	22.2±0.28	1.704±0.470
2	1	1	2	1	15.4±0.33	1.162±0.277
3	1	2	3	3	25.8±0.27	1.950±0.339
4	2	2	1	1	16.7±0.27	1.234±0.279
5	2	3	2	3	15.0±0.26	1.150±0.292
6	2	1	3	2	12.0±0.07	0.840±0.070
7	3	1	1	3	11.2±0.32	0.778±0.239
8	3	2	2	2	15.2±0.46	1.170±0.283
9	3	3	3	1	15.6±0.23	1.162±0.286

The arrangements of columns A, B, C and D were decided by orthogonal design for 4 (factor) × 9 (run number), every row of a run number represents one experiment replicate; every run was carried out five times. Values are mean±S.D. of five determinations

Analytical methods

Samples collected at various intervals from shake flasks were filtered using 40-mesh stainless sieve and the mycelium was harvested. The mycelium was washed using deionized water, dried to constant mass at 80 °C and then weighed. A mass of 1 g of dry mycelia were hydrolyzed for 4 h at 40 °C with 30 mL of 1 mol/L NaOH. The pellets of mycelia were removed through suction filtration and washed 3 times with 20 mL of deionized water. The filtrate was combined to further analyse the content of the polysaccharides. The level of polysaccharides was equal to gross sugar content minus reducing sugar content. Gross sugar content and reducing sugar content were determined as anthrone sulphate

method (14) and 3,5-dinitrosalicylic acid method (15), respectively. Gross protein content was determined by biuret reaction (16).

Statistical analysis

The analyses were made at least in quintuplicate and the results presented were expressed as mean \pm S.D. Statistical analysis was performed using one way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT). P-values ≤ 0.05 were considered significant and ≤ 0.01 were extremely significant.

Results and Discussion

Effect of culture temperature and initial pH

To investigate the effect of different temperature on mycelial growth and polysaccharide production, experiments were carried out at 20 to 35 °C. Data in Table 3 show that dry mycelial mass and polysaccharide yield did not change significantly when the temperature was set from 25 to 30 °C (23.9–24.8 and 1.78–1.96 g/L, respectively, $p > 0.05$). However, the two responses at 25–30 °C were remarkably higher than those at temperature higher than 35 °C or lower than 20 °C ($p \leq 0.01$), so the temperature of following experiments was set at 28 °C.

Table 3. Effect of temperature on mycelial growth and polysaccharides produced by *T. aurantialba*

Temperature °C	γ (dry mycelia) g/L	γ (mycelial polysaccharides) g/L
20	20.3 \pm 1.8	1.12 \pm 0.16
25	23.9 \pm 2.4	1.78 \pm 0.21
28	24.8 \pm 1.9	1.96 \pm 0.18
30	24.3 \pm 1.3	1.84 \pm 0.23
35	18.7 \pm 2.7	0.96 \pm 0.26

The initial pH of the culture medium was adjusted to different levels with 1 mol/L HCl or 1 mol/L NaOH solution after the culture medium was sterilized. The effects of initial pH on mycelial growth and polysaccharide yield are shown in Table 4. Statistical analysis showed that no significant difference existed when pH was adjusted from 5.5 to 7.5 (23.1–24.5 and 1.87–1.97 g/L, respectively, $p > 0.05$).

Table 4. Effect of initial pH on mycelial growth and polysaccharides produced by *T. aurantialba*

Initial pH	γ (dry mycelia) g/L	γ (mycelial polysaccharides) g/L
5.5	24.3 \pm 1.9	1.96 \pm 0.19
6.0	24.5 \pm 2.1	1.94 \pm 0.21
6.5	23.9 \pm 1.6	1.89 \pm 0.21
7.0	24.4 \pm 1.6	1.97 \pm 0.18
7.5	23.1 \pm 2.4	1.93 \pm 0.18

Effect of carbon source

To find the most suitable carbon source for mycelial growth and polysaccharide production in *T. aurantialba*, various types of carbon sources including five hexoses (glucose, mannose, altrose, galactose and fructose), four pentoses (lyxose, xylose, ribose and xylulose), four disaccharides (maltose, sucrose, lactose and cellobiose) and four polysaccharides (starch, dextrin, cellulose and xylan) (40 g/L) were supplemented instead of glucose as the carbon source in the basal medium. The results are shown in Table 5. Data in Table 5 show that the dry mycelia mass and polysaccharide yield were not significantly different from each other ($p > 0.05$) using all tested six-carbon aldoses as carbon sources (18.5–20.1 and 1.06–1.14 g/L, respectively), but when compared with ketose (fructose) (15.3 g/L and 0.92 g/L, respectively), their dry mycelia mass and polysaccharide yield were significantly higher than when fructose was used as carbon source ($p \leq 0.01$). Similar results were given when pentoses were used.

Comparing the mycelia mass and polysaccharide yield using five-carbon aldose (lyxose, xylose and ribose) as a carbon source with six-carbon aldose, and five-carbon ketose (xylulose) with six-carbon ketose, respectively, it was obvious that: (i) the dry mycelia mass and polysaccharide yield were both higher when the five-carbon aldose was used as carbon source than when the six-carbon aldose was used; (ii) the dry mycelia mass and polysaccharide yield were both higher when the five-carbon ketose were used as carbon source than when the six-carbon ketose was used; and (iii) the five-carbon aldose was the optimum carbon source among all the tested monosaccharides.

Table 5. Effect of carbon sources on mycelial growth and polysaccharides produced by *T. aurantialba*

	Carbon source	γ (dry mycelia) g/L	γ (mycelial polysaccharides) g/L
aldose	glucose	20.1 \pm 2.5	1.14 \pm 0.16
	mannose	19.6 \pm 3.5	1.12 \pm 0.14
	altrose	18.5 \pm 2.9	1.08 \pm 0.21
	galactose	19.4 \pm 2.7	1.06 \pm 0.18
	fructose	(15.3 \pm 1.8)*	(0.92 \pm 0.12)*
pentose	lyxose	24.1 \pm 2.3	1.86 \pm 0.15
	xylose	25.3 \pm 1.7	1.98 \pm 0.14
	ribose	24.8 \pm 2.7	1.92 \pm 0.18
	xylulose	(19.8 \pm 2.6)*	(1.54 \pm 0.21)*
disaccharide	maltose	19.8 \pm 1.8	1.09 \pm 0.17
	sucrose	(17.6 \pm 1.7)*	(0.97 \pm 0.13)*
	lactose	19.8 \pm 1.9	1.15 \pm 0.16
polysaccharide	cellobiose	20.1 \pm 2.3	1.06 \pm 0.13
	starch	14.7 \pm 3.5	0.89 \pm 0.21
	dextrin	14.1 \pm 2.8	0.87 \pm 0.19
	cellulose	18.3 \pm 2.5	1.04 \pm 0.17
	xylan	(23.6 \pm 1.4)*	(1.35 \pm 0.13)*

In contrast with other carbon sources in the relative group: * $p < 0.01$

Four kinds of disaccharides were employed in the test. It can be demonstrated from Table 5 that the dry mycelia mass and polysaccharide yield were not significantly different from each other ($p>0.05$) when maltose, lactose and cellobiose were used as carbon sources (19.8–20.1 and 1.06–1.15 g/L, respectively). But the dry mycelia mass and polysaccharide yield using sucrose as carbon source (17.6 and 0.97 g/L, respectively) were lower than those using other disaccharides as carbon sources, which was highly significant ($p\leq 0.01$). The molecule of sucrose consisted of one molecule of aldose and ketose, and other three kinds of molecules were made of two molecules of aldose. This further proved that the optimum carbon source for *T. aurantialba* was an aldose rather than ketose.

Four kinds of polysaccharides were used in the test. The result indicated that the dry mycelia mass and polysaccharide yield by *T. aurantialba* when starch and dextrin were used as carbon sources (14.7, 14.1 and 0.89, 0.87 g/L, respectively) were lower than when cellulose was used (18.3 g/L and 1.04 mg/L, respectively, $p\leq 0.01$), which were in turn lower than when xylan was used (23.6 and 19.5 g/L, respectively, $p\leq 0.01$). Comparing xylan with xylose (25.3 and 1.98 g/L, respectively), their dry mycelia mass and polysaccharide yield were not significantly different ($p>0.05$).

It could be concluded that the highest mycelia mass and polysaccharide yield were obtained in xylose or xylan medium. In order to meet the requirements for mass production, xylan was chosen as carbon source in subsequent experiments, which could reduce the production cost of *T. aurantialba* fermentation since the market price of xylan is only 1/3 of that of xylose.

Effect of initial xylan concentration

To study the effect of different concentrations of xylan on the mycelial growth and polysaccharide production, experiments were carried out with the initial xylan concentrations ranging from 5 to 50 g/L. Data in Table 6 show that optimal xylan concentration was 40 g/L (23.6 and 1.95 g/L, respectively). However, no further increases of mycelia mass and polysaccharide yield were observed when xylan concentration exceeded 40 g/L.

Table 6. Effect of xylan concentration on the mycelia and polysaccharides produced by *T. aurantialba*

$w(\text{xylose})$ %	γ (dry mycelia) g/L	γ (mycelial polysaccharides) g/L
0.5	13.4±1.9	0.24±0.11
1.0	15.4±2.1	0.26±0.13
1.5	16.3±1.7	0.37±0.18
2.0	17.3±1.6	0.57±0.16
2.5	18.4±1.8	1.06±0.14
3.0	20.9±1.6	1.57±0.17
3.5	22.2±1.8	1.80±0.13
4.0	23.6±2.2	1.95±0.15
4.5	23.4±1.6	1.94±0.16
5.0	23.8±1.2	1.98±0.18

Effect of nitrogen source

To investigate the effect of nitrogen source on mycelial growth and polysaccharide production, cells were cultivated in the medium containing various nitrogen sources, where each nitrogen source was added to the basal medium at a concentration of 10 g/L. Results in Table 7 show that the dry mycelia mass and polysaccharide yield were not significantly different from each other for the organic nitrogen sources (24.1–25.8 and 1.86–1.98 g/L, respectively, $p>0.05$). In comparison with organic nitrogen sources, a relatively lower mycelial growth and polysaccharide production was observed when inorganic nitrogen sources were used (11.4–18.1 and 1.02–1.54 g/L, respectively, $p\leq 0.01$). Peptone was chosen as nitrogen source in subsequent experiments, because peptone could be obtained conveniently and is widely used in the industry.

Table 7. Effect of nitrogen sources on mycelial growth and polysaccharides produced by *T. aurantialba*

Nitrogen source	γ (dry mycelia) g/L	γ (mycelial polysaccharides) g/L
KNO ₃	16.8±1.6	1.04±0.16
(NH ₄) ₂ SO ₄	11.4±3.5	1.02±0.17
CO(NH ₂) ₂	18.4±2.4	1.54±0.16
Peptone	25.1±2.2	1.97±0.15
Yeast extract	24.8±1.9	1.92±0.12
Bear powder	24.1±2.3	1.86±0.15
Tryptone	25.3±1.9	1.96±0.19
Casamino acid	25.8±1.8	1.98±0.16

Effect of complex nutrition sources

To study the effect of complex nutrition source on mycelial growth and polysaccharide production, cultures were carried out with the medium containing various complex nutrition sources (10 g/L). Table 8 shows that among four kinds of complex nutrition sources examined, a high level of the responses was obtained when wheat bran and corn powder were used as complex nutrition sources (21.4 and 19.8 g/L, and 1.58 and 1.47 g/L, respectively).

Table 8. Effect of complex nutrition source on mycelial growth and polysaccharides produced by *T. aurantialba*

Growth factor	γ (dry mycelia) g/L	γ (mycelial polysaccharides) g/L
Control	15.6±1.6	0.92±0.16
Potato	17.6±3.5	1.01±0.18
Corn powder	19.8±2.5	1.47±0.16
Wheat bran	21.4±2.2	1.58±0.13
Rice bran	14.8±2.6	0.89±0.21

Effect of mineral salts

The influence of mineral salts on mycelial growth and polysaccharide yield was examined by supplementation of various mineral sources into the medium (Table 9). Among the various mineral salts examined, $MgSO_4$ and KH_2PO_4 could slightly increase the dry mycelia mass and polysaccharide yield, compared to control. Other mineral salts show negative influence on the dry mycelia mass and polysaccharide yield. Hence, $MgSO_4$ and KH_2PO_4 were supplied at concentrations of 0.6 and 1.2 g/L, respectively, while other mineral salts were not added. These results show little difference from *Psathyrella atro-umbonata* that could obtain a high level of mycelial growth in media containing calcium and magnesium (17).

Table 9. Effect of mineral salts on mycelial growth and polysaccharides produced by *T. aurantialba*

Mineral salt	γ g/L	γ (dry mycelia) g/L	γ (mycelial polysaccharides) g/L
Control	–	23.8±1.8	1.80±0.18
$MgSO_4$	0.6	24.1±2.1	1.88±0.21
KH_2PO_4	1.2	24.7±1.6	1.96±0.19
$CoCl_2$	0.2	21.4±1.6	1.64±0.21
$FeSO_4$	0.2	22.3±1.6	1.75±0.17
$CaCl_2$	0.2	23.1±1.8	1.74±0.24
$CuSO_4$	0.2	18.7±2.7	0.96±0.26
$CrCl_3$	0.05	16.2±2.4	1.42±0.21
$MnCl_2$	0.05	17.6±1.8	1.53±0.19

Optimization results by the orthogonal matrix method

During the optimized experiments, the fermentations were carried out in a rotary shaker incubator at 150 rpm and 28 °C for 7 days. The initial pH of the medium was adjusted to 6.0. The experimental data of dry mycelia mass and polysaccharide yield are shown in the last two columns in Table 2. The effect of culture media on mycelial growth and polysaccharide production was calculated and the results (in Table 10) showed that there were significant differences in the tested concen-

tration of xylan, peptone and wheat bran ($p \leq 0.01$), however, no significant difference was observed in the tested concentration of corn powder ($p > 0.05$). According to the order of magnitude of R, the order of effect of all factors on mycelial growth and polysaccharide yield was xylan > peptone > wheat bran > corn powder. This result pointed out that the effect of xylan as carbon source was more important than that of other nutrients. It could be demonstrated that the optimal medium was (in g/L): xylan 40, peptone 10, wheat bran 20, corn powder 20, KH_2PO_4 1.2 and $MgSO_4 \cdot 7H_2O$ 0.6.

Dry mycelia mass and yield of polysaccharides in a 50-litre stirred-tank bioreactor

Fig. 1 shows typical time courses of mycelial growth and polysaccharide production in 50-litre stirred-tank bioreactor. Under the optimal culture conditions (in g/L): xylan 40, peptone 10, bran 20, corn powder 20, KH_2PO_4 1.2 and $MgSO_4 \cdot 7H_2O$ 0.6, the concentration of residual sugar in broth decreased to minimum (3.1 g/L) within the first 70 h. The time for polysaccharide production and mycelial growth was out of step. The amount of polysaccharides kept rising from 50 to 132 h and reached the maximum production (3.01 g/L) at 132 h, while the

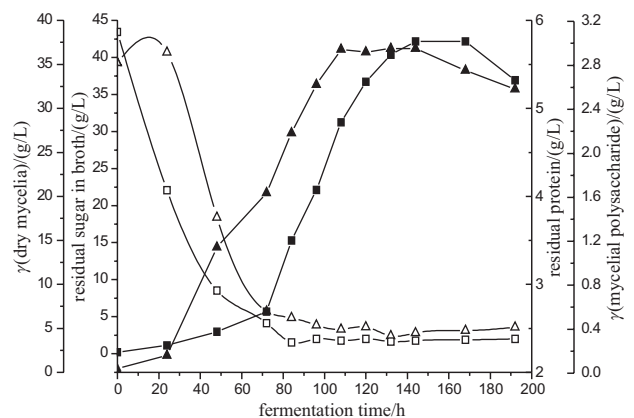


Fig. 1. Time profile of sugar consumption (Δ), protein consumption (\square), dry mycelia mass (\blacktriangle) and polysaccharide production (\blacksquare) of *T. aurantialba* in 50-litre stirred-tank bioreactor under the optimized culture conditions. Fermentations were carried out for 192 h at 28 °C

Table 10. Analysis of media on dry mycelia mass and polysaccharide production in shake flask culture of *Tremella aurantialba* with orthogonal projects

	γ (dry mycelia)/(g/L)				γ (mycelial polysaccharides)/(g/L)			
	A	B	C	D	A	B	C	D
K1	63.3±13.5	38.4±0.54	50.1±14.1	47.1±1.8	4.818±1.02	2.781±0.53	3.717±1.179	3.57±0.129
K2	43.5±6.0	57.6±1.44	45.6±12.9	49.5±13.5	3.21±0.513	4.35±0.11	3.48±0.081	3.714±1.20
K3	42.0±6.3	52.8±1.02	53.1±18.0	51.9±19.2	3.111±0.573	4.017±0.81	3.96±1.449	3.87±1.521
k1	(21.1±4.5)*	12.8±0.18	16.7±4.7	15.9±0.6	1.606±0.34	0.927±0.18	1.239±0.393	1.19±0.043
k2	14.5±2.0	(19.2±0.48)*	15.2±4.3	16.5±4.5	1.07±0.171	(1.45±0.35)*	1.16±0.027	1.238±0.40
k3	14.0±2.1	17.6±0.34	17.7±6.0	17.3±6.4	1.037±0.191	(1.339±0.26)*	(1.32±0.483)*	1.29±0.507
R	7.1±6.6	6.4±0.66	2.5±10.3	1.4±7.0	0.569±0.531	0.523±0.53	0.16±0.51	0.10±0.55
Optimal level	1	2	3	3	1	2	3	3

In contrast with other levels of the same factor: * $p < 0.01$

dry mycelia mass kept rising from 25 to 108 h and reached the maximum mass (36.8 g/L) at 108 h. It was obvious that the time for polysaccharide production lags behind that for mycelial growth.

Conclusion

To date, many researchers have focused on the polysaccharide production from mushroom by submerged cultures (18,19). Statistical optimization method could overcome the limitations of classic empirical methods and some inevitable test errors. In this work it was proved to be a powerful tool for the optimization of the culture conditions for *T. aurantialba*. The optimization strategy established in this study may be worth applying in other mushroom fermentation processes for enhanced production of mushroom polysaccharides, particularly for those with potential industrial application. In this study, the orthogonal matrix method was proposed to study the influence of controlled factors in a multivariable system. The optimal media were found to be composed of (in g/L): xylan 40, peptone 10, bran 20, corn powder 20, KH_2PO_4 1.2 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.6 for the enhanced production of polysaccharides and mycelial mass. Under optimal culture conditions, the maximum dry mycelia mass and polysaccharide yield in a 50-litre stirred-tank bioreactor reached 36.8 and 3.01 g/L, respectively. Simultaneously, the time profile of fermentation in a 50-litre stirred-tank bioreactor showed that the time for polysaccharide production and mycelial growth was out of step and the time for polysaccharide production lags behind that for mycelial growth. The results of this study provided useful information and reference for the optimization of medium composition for the other submerged mushroom fermentation processes.

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