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Optimization of Medium by Orthogonal Matrix Method for Submerged Mycelial Culture and Exopolysaccharide Production in *Collybia maculata*

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Abstract

Optimization of submerged culture conditions for the production of mycelial growth and exopolysaccharides (EPSs) by *Collybia maculata* was investigated. The optimum temperature and the initial pH for EPS production in a shake-flask culture of *C. maculata* were found to be 20°C and 5.5, respectively. Among the various medium's constituents examined, glucose, Martone A-1, K_2HPO_4, and $CaCl_2$ were the most suitable carbon, nitrogen, and mineral sources for EPS production, respectively. The optimum concentration of the medium's ingredients determined using the orthogonal matrix method was as follows: 30 g/L of glucose, 20 g/L of Martone A-1, 1 g/L of K_2HPO_4, and 1 g/L of $CaCl_2$. Under the optimized culture conditions, the maximum concentration of EPSs in a 5-L stirred-tank reactor was 2.4 g/L, which was approximately five times higher than that in the basal medium. A comparative fermentation result showed that the EPS productivity in an airlift reactor was higher than that in the stirred-tank reactor despite the lower mycelial growth rate. The specific productivities and the yield coefficients in the airlift reactor were higher than those in the stirred-tank reactor even though the volumetric productivities were higher in the stirred-tank reactor than in the airlift reactor.

Index Entries: *Collybia maculata*; exopolysaccharide; optimization; orthogonal matrix method; submerged culture.

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Introduction

A number of microbial polysaccharides from higher fungi such as Basidiomycetes and Ascomycetes have been used to maintain health and increase longevity in humans *(1–3)*. It is widely known that most polysaccharides produced by submerged culture of mushrooms induce various biologic activities, including immunostimulating, antitumor, and hypoglycemic activities *(4–7)*.

Collybia maculata is an edible mushroom, which belongs to the phylum Basidiomycota, the class Basidiomycetes, and the family Tricholomataceae. Although many investigators have attempted to obtain optimum submerged culture conditions for exopolysaccharide (EPS) production from several mushrooms, to the best of our knowledge, the nutritional requirements and environmental conditions for submerged culture of *C. maculata* have not been demonstrated so far *(8,9)*.

In the present study, the orthogonal matrix method was employed to study the relationships between the components of the medium and their effects on mycelial growth and EPS production. In comparison with other statistical methods, this method is a sophisticated and cost-effective optimization strategy that can be used to assign experimental factors in experimental trials. The objective of the present study was to optimize the medium's constituents for mycelial growth and EPS production by *C. maculata* using the orthogonal matrix method.

Materials and Methods

Microorganism and Media

C. maculata used in our culture collection was isolated from a mountainous district in Korea. The stock culture was maintained on potato dextrose agar (PDA) slants and subcultured once a month. Slants were incubated at 25°C for 6 d and then stored at 4°C. The seed culture was grown in 250-mL flasks containing 50 mL of Mushroom Complete Medium (MCM) (2% glucose, 0.2% yeast extract, 0.2% peptone, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% K_2HPO_4, 0.046% KH_2PO_4) at 25°C on a rotary shaker incubator at 150 rpm for 3 d. The shake-flask culture was grown in 250-mL flasks containing 50 mL of MCM. The bioreactor experiments were performed in a 5-L stirred-tank reactor and in an airlift reactor containing 3 L of the optimum medium (3% glucose, 2% Martone A-1, 0.1% $CaCl_2$, and 0.1% K_2HPO_4).

Preparation of Inoculum and Flask Cultures

C. maculata was initially grown on PDA medium in a Petri dish and then transferred into the seed culture medium by punching out 5 mm of the agar plate culture with a self-designed cutter. The seed culture was grown in 250-mL flasks containing 50 mL of basal medium at 25°C on a rotary shaker incubator at 150 rpm for 3 d. The flask culture experiments were

performed in 250-mL flasks containing 50 mL of the media after inoculating with 4% (v/v) of the seed culture. All experiments were carried out at least in duplicate to ensure reproducibility.

Orthogonal Matrix Method

The optimum levels of the four significant experimental factors were searched according to the orthogonal matrix method, in which three levels, four factors, and nine experiments $\{L_9(3^4)\}$ of experimental design were employed. To reach the same results as those of the orthogonal matrix method, $3^4 \times 2$ replicates, 162 experiments are necessary to achieve the experimental goals for full-factor experimental projects.

Fermentation in Bioreactor

The fermentation medium was inoculated with 4% (v/v) of the seed culture and then cultivated at 20°C in a 5-L stirred-tank reactor (KoBioTech, Seoul, Korea) equipped with pH and dissolved oxygen electrodes. Unless otherwise specified, fermentations were performed under the following conditions: temperature, 20°C; aeration rate, 2 vvm; agitation speed, 150 rpm; initial pH, 5.5; working volume, 3 L. A comparative fermentation was also conducted in a 5-L airlift reactor (Best Korea, Seoul, Korea) under the same operating conditions as in the stirred-tank reactor.

Analytical Methods

Samples collected at various intervals from the shake-flask cultures were centrifuged at 9000*g* for 15 min, and the resulting supernatant was filtered through a Whatman No. 2 filter paper (Whatman, Maidstone, England). The resulting culture filtrate was mixed with 4 vol of absolute ethanol, stirred vigorously, and left overnight at 4°C. The precipitated EPS was centrifuged at 9000*g* for 15 min, and the supernatant was discarded. The precipitate of pure EPS was lyophilized and its weight estimated. The dry weight of mycelium was measured after repeated washing of the mycelial pellets with distilled water and drying overnight at 70°C to a constant weight. Residual sugar concentration was quantitatively analyzed by high-performance liquid chromatography (Shimadzu, Osaka, Japan) using an Aminex HPX-42C column (Bio-Rad, Hercules, CA) equipped with a refractive index detector.

Estimation Procedures in Fermentation Kinetics

The specific growth rate was determined according to the following equation:

$$\mu\ (d^{-1}) = (1/X)(dX/dt)$$

in which X is the cell concentration (g/L) and t is the culture time (d) *(10,11)*.

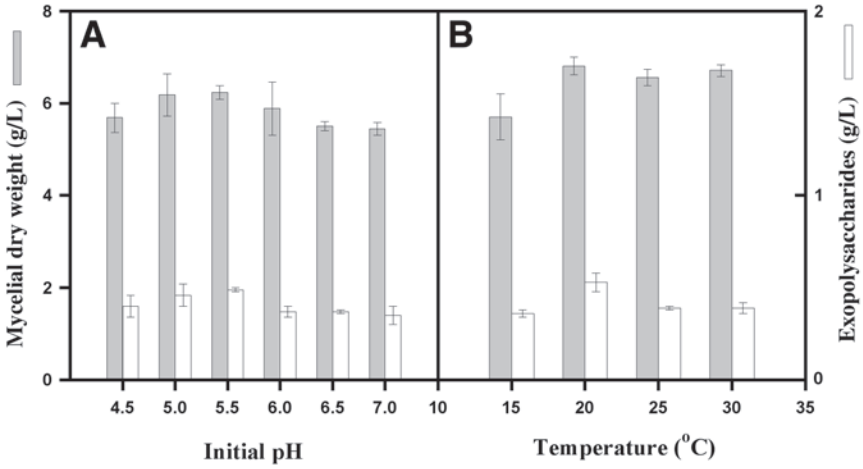

Fig. 1. Effect of **(A)** initial pH and **(B)** temperature on mycelial growth and EPS production in shake-flask cultures of *C. maculatas*.

The specific consumption rate of substrate was estimated according to the following equation:

$$Q_{S/X}\,(\mathrm{g}/[\mathrm{g}\cdot\mathrm{d}]) = (1/X)(dS/dt)$$

in which S is the substrate concentration (g/L) and t is the culture time (d).

The specific production rate of EPS was calculated by the following equation:

$$P_{P/X}\,(\mathrm{g}/[\mathrm{g}\cdot\mathrm{d}]) = (1/X)(dP/dt)$$

in which P is the product concentration (g/L) and t is the culture time (d).

The EPS yield on substrate was calculated by the following equation:

$$Y_{P/S}\,(\mathrm{g}/\mathrm{g}) = (dP/dt)/(dS/dt)$$

Results and Discussion

Effect of Initial pH and Temperature

To investigate the effect of initial pH on mycelial growth and EPS production, *C. maculata* was cultivated in MCM medium with different initial pH values (4.0–7.0) in the shake-flask culture. As shown in Fig. 1A, the optimum pH for both mycelial growth and EPS production was 5.5. This same result indicates that higher fungi have relatively acidic pH optima during their submerged cultures *(12,13)*.

To determine the optimum temperature for mycelial growth and EPS production, *C. maculata* was cultivated at various temperatures ranging from 15 to 30°C. The results in Fig. 1B indicate that the optimum temperature for both the highest mycelial growth and EPS production was 20°C. It has been reported that higher fungi usually require long periods for their

Table 1
Effect of Carbon Sources on Mycelial Growth
and EPS Production in Shake-Flask Cultures of *C. maculata* TG-1[a]

Carbon source (2%)	Mycelial biomass (g/L)	EPS (g/L)	Final pH
Fructose	7.74 ± 0.28	0.22 ± 0.00	2.53
Glucose	7.37 ± 0.09	0.29 ± 0.03	2.41
Lactose	4.03 ± 0.21	0.13 ± 0.01	4.11
Maltose	6.01 ± 0.31	0.31 ± 0.03	2.65
Mannitol	6.04 ± 0.09	0.11 ± 0.03	2.76
Sucrose	6.91 ± 0.01	0.21 ± 0.05	2.55
Xylose	7.26 ± 0.32	0.28 ± 0.04	2.58

[a]Fermentations were carried out for 6 d at 20°C with an initial pH of 5.5.

submerged cultures, exposing them to contamination risk, and, thus, this low temperature optimum is regarded as a favorable physiologic property *(12,14)*.

Nutritional Requirements of C. maculata

The influence of carbon sources on mycelial growth and EPS production by *C. maculata* was studied in medium containing 2% (v/v) of different carbon sources. Although mycelial growth was the most favorable in fructose medium, maximum EPS production was achieved in glucose medium (Table 1) *(15)*.

To select a suitable nitrogen source for mycelial growth and EPS production, various nitrogen sources were provided at a concentration of 0.4% (v/v) omitting yeast extract and peptone used in the basal medium. Among 12 nitrogen sources examined, polypeptone, casein peptone, and Martone A-1 were efficient for mycelial growth of *C. maculata* (Table 2), and maximum EPS production was achieved in medium containing Martone A-1. In comparison with organic nitrogen sources, inorganic nitrogen sources gave rise to relatively lower mycelial growth and EPS production *(12,16–18)*.

The influence of mineral ions on mycelial growth and EPS production was also examined at a concentration of 7 m*M*. Among the various mineral ions examined, K_2HPO_4 and $CaCl_2$ had beneficial effects on mycelial growth and EPS production. The maximum mycelial growth and EPS production were achieved when K_2HPO_4 and $CaCl_2$ were simultaneously employed (Table 3). It has been reported that calcium and potassium ions are recognized as favorable mineral ions for mycelial growth and EPS production in several mushroom fermentations *(17–20)*.

Orthogonal Matrix Results

Orthogonal projects, as a result of the suitable design of factors, can give effective responses in many system optimizations *(21–23)*.

Table 2
Effect of Nitrogen Sources on Mycelial Growth and EPS Production in Shake-Flask Cultures of *C. maculata* TG-1[a]

Nitrogen source (0.4%)	Mycelial biomass (g/L)	EPS (g/L)	Final pH
Ammonium chloride	3.38 ± 0.32	0.52 ± 0.04	1.41
Ammonium citrate	5.27 ± 0.28	0.22 ± 0.01	3.03
Ammonium nitrate	2.70 ± 0.10	0.31 ± 0.05	1.54
Ammonium phosphate	3.50 ± 0.04	0.31 ± 0.05	1.99
Casein peptone	8.31 ± 0.14	0.39 ± 0.01	1.85
Martone A-1	9.10 ± 0.16	0.60 ± 0.04	2.04
Meat peptone	6.99 ± 0.05	0.36 ± 0.04	1.92
Polypeptone	7.87 ± 0.21	0.39 ± 0.01	1.95
Sodium nitrate	1.88 ± 0.08	0.25 ± 0.05	2.77
Soy peptone	5.58 ± 0.20	0.35 ± 0.03	2.40
Tryptone	7.20 ± 0.30	0.53 ± 0.04	2.13
Yeast extract	6.42 ± 0.22	0.23 ± 0.05	2.05

[a]Fermentations were carried out for 6 d at 20°C with an initial pH of 5.5.

Table 3
Effect of Mineral Sources on Mycelial Growth and EPS Production in Shake-Flask Cultures of *C. maculata* TG-1[a]

Mineral source (7 m*M*)	Mycelial biomass (g/L)	EPS (g/L)	Final pH
Control[b]	6.42 ± 0.32	0.21 ± 0.03	1.79
$Na_2H_2PO_4$	6.08 ± 0.20	0.26 ± 0.00	1.87
$MgSO_4 \cdot 7H_2O$	6.63 ± 0.40	0.17 ± 0.01	1.84
$CaCl_2$	8.09 ± 0.43	0.24 ± 0.02	1.95
K_2HPO_4	7.03 ± 0.27	0.37 ± 0.09	1.88
KH_2PO_4	6.35 ± 0.00	0.15 ± 0.03	1.96
KI	2.91 ± 0.01	0.48 ± 0.04	3.06
KCI	5.82 ± 0.15	0.20 ± 0.09	1.87
$MnCl_2 \cdot 4H_2O$	6.90 ± 0.67	0.18 ± 0.06	2.28
$CaCl_2 + K_2HPO_4$	8.56 ± 0.48	0.80 ± 0.02	2.01

[a]Fermentations were carried out for 6 d at 20°C with an initial pH of 5.5.
[b]Control means the medium containing 2% carbon sources and 0.4% nitrogen sources without the addition of any mineral sources.

Table 4 illustrates the factor allocations of the orthogonal matrix, in which the letters A, B, C, and D represent the glucose, Martone A-1, $CaCl_2$, and K_2HPO_4 concentration, respectively. In the course of optimization experiments, the fermentation temperature, initial pH, agitation rate, and culture period were fixed at 20°C, 5.5, 150 rpm, and 6 d, respectively. The effect of those factors on mycelial growth and EPS production was calculated and the results are shown in Table 5. To obtain the optimization levels or composition of each factor, the intuitive analysis was conducted based on sta-

Table 4
Experimental Factors and Their Levels for Orthogonal Matrix[a]

Level	A (%)	B (%)	C (%)	D (%)
1	2	1	0.05	0.05
2	3	2	0.10	0.10
3	4	3	0.15	0.15

[a]A, B, C, and D represent the concentration of glucose, Martone A-1, $CaCl_2$, and K_2HPO_4.

Table 5
Assignment of Factors and Levels in Optimization Experiments Using L_9 (3^4) Orthogonal Matrix to Mycelial Growth and EPS Production by *C. maculata* TG-1[a]

Run	A (%)	B (%)	C (%)	D (%)	Mycelial biomass (g/L)	EPS (g/L)
1	1 (2)	1 (1)	1 (0.05)	1 (0.05)	11.84 ± 0.00	1.30 ± 0.13
2	1 (2)	2 (2)	2 (0.10)	2 (0.10)	13.82 ± 0.30	3.20 ± 0.00
3	1 (2)	3 (3)	3 (0.15)	3 (0.15)	10.75 ± 0.45	1.70 ± 0.50
4	2 (3)	1 (1)	2 (0.10)	3 (0.15)	12.91 ± 1.15	2.03 ± 0.13
5	2 (3)	2 (2)	3 (0.15)	1 (0.05)	15.35 ± 1.69	2.11 ± 0.13
6	2 (3)	3 (3)	1 (0.05)	2 (0.10)	14.43 ± 0.71	2.10 ± 0.50
7	3 (4)	1 (1)	3 (0.15)	2 (0.10)	13.84 ± 0.20	2.40 ± 0.75
8	3 (4)	2 (2)	1 (0.05)	3 (0.15)	15.74 ± 0.52	2.50 ± 0.50
9	3 (4)	3 (3)	2 (0.10)	1 (0.05)	12.54 ± 0.76	1.30 ± 0.25

[a]The arrangement of columns A, B, C, and D was decided by orthogonal design for 4 (factor) × 9 (run number); every row of run number represents one experimental replicate and every run was replicated twice. Values are the mean ± SD of double determinations.
A, B, C, and D represent the concentration of glucose, Martone A-1, $CaCl_2$, and K_2HPO_4.

tistical calculation using the data in Table 6 and Fig. 2. The optimization results were as follow: (1) to obtain high mycelial growth, optimum compositions were 3% glucose, 2% Martone A-1, 0.05% $CaCl_2$, and 0.1% K_2HPO_4; (2) to obtain maximum EPS production, optimum compositions were 3% glucose, 2% Martone A-1, 0.1% $CaCl_2$, and 0.1% K_2HPO_4.

Fermentation Results

The typical time courses of mycelial growth and EPS production in a 5-L stirred-tank reactor under the basal medium and optimized medium are shown in Fig. 3A,B. In the basal medium, the maximum mycelial biomass and EPS concentration was 7.3 and 0.5 g/L at 6 d, respectively. The mycelial growth rapidly increased for the first 3 d with a corresponding depletion of glucose, and the initial pH of the fermentation broth sharply decreased from 5.0 to 2.6 (Fig. 3A). In the optimized medium, the maximum EPS production was 2.4 g/L after 5 d, which was five times higher than that

Table 6
Analysis of Effect of Media on Mycelial Growth
and EPS Production in Shake-Flask Cultures of *C. maculata* TG-1 Using Orthogonal Matrix

	Mycelial biomass (g/L)				EPS (g/L)			
	A	B	C	D	A	B	C	D
K_1	36.41 ± 0.75[a]	38.59 ± 1.35	42.01 ± 1.23	39.73 ± 2.45	6.20 ± 0.63	5.73 ± 1.00	5.90 ± 1.12	4.71 ± 0.50
K_2	42.69 ± 3.55	44.91 ± 2.51	39.27 ± 2.21	42.09 ± 1.21	6.24 ± 0.75	7.81 ± 0.62	6.53 ± 0.37	7.70 ± 1.25
K_3	42.12 ± 1.48	37.72 ± 1.92	39.94 ± 2.34	39.40 ± 2.12	6.20 ± 1.50	5.10 ± 1.25	6.21 ± 1.38	6.23 ± 1.12
k_1	12.14 ± 0.25[b]	12.86 ± 0.45	14.00 ± 0.41	13.24 ± 0.82	2.07 ± 0.21	1.91 ± 0.33	1.97 ± 0.38	1.57 ± 0.17
k_2	14.23 ± 1.18	14.97 ± 0.84	13.09 ± 0.74	14.03 ± 0.40	2.08 ± 0.25	2.60 ± 0.21	2.18 ± 0.13	2.57 ± 0.42
k_3	14.04 ± 0.49	12.57 ± 0.64	13.31 ± 0.78	13.13 ± 0.71	2.07 ± 0.50	1.70 ± 0.42	2.07 ± 0.46	2.08 ± 0.38
R	2.09 ± 0.93[c]	2.40 ± 0.39	0.91 ± 0.37	0.90 ± 0.41	0.01 ± 0.29	0.90 ± 0.21	0.21 ± 0.33	1.00 ± 0.25
Optimum level	2	2	1	2	2	2	2	2

[a] K^A_i = Σ mycelial yield at A_i. Values are the mean ± SD of double determinations.
[b] $k^A_i = k^A_i/3$. Values are the mean ± SD of double determinations.
[c] $R^A_i = \max\{k^A_i\} - \min\{k^A_i\}$. Values are the mean ± SD of double determinations.

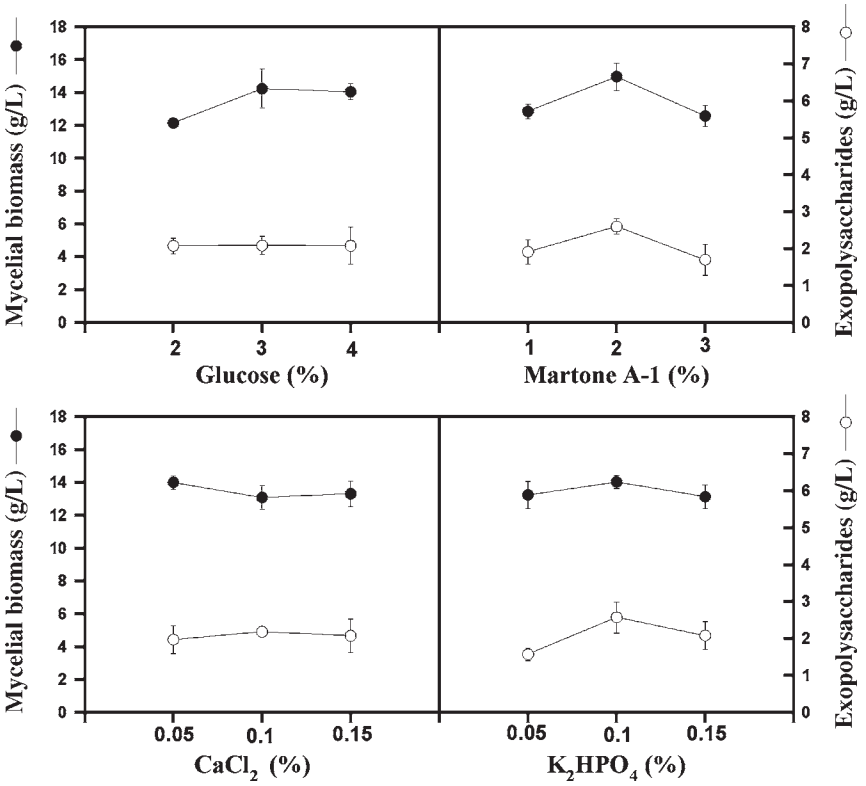

Fig. 2. Orthogonal matrix analysis of the relationship between media and mycelial growth and EPS production in shake-flask cultures of *C. maculata*. Experimental data are the mean ± SD of double determinations.

at the basal medium level (Fig. 3B). The kinetic parameters of *C. maculata* with basal medium and optimum medium in a 5-L stirred-tank reactor are comparatively illustrated in Table 7. The specific growth rates (μ) in the basal medium and in the optimized medium were 0.161 and 0.23 d^{-1}, respectively. The EPS yield in optimized medium (0.079 g/g) was about 10-fold higher than that in basal medium (0.008 g/g). For a comparative study, fermentation was also carried out in a 5-L airlift reactor under the same operating conditions as in the stirred-tank reactor. In stirred-tank reactors, the shear stress usually causes undesired effects on mycelial morphology, product formation, and product yields. Because airlift reactors do not require mechanical agitation and do not have mechanical parts, the shear stress is considerably less than that in stirred-tank reactors *(24,25)*. It was observed that the maximum mycelial biomass and EPS concentration in the airlift reactor was 8.1 g/L at d 4 and 2.1 g/L at d 3, respectively (Fig. 3C). The specific productivities and the yield coefficients in the airlift reactor were higher than those in the stirred-tank reactor, even though the volumetric productivities were higher in the stirred-tank reactor than in the airlift reactor.

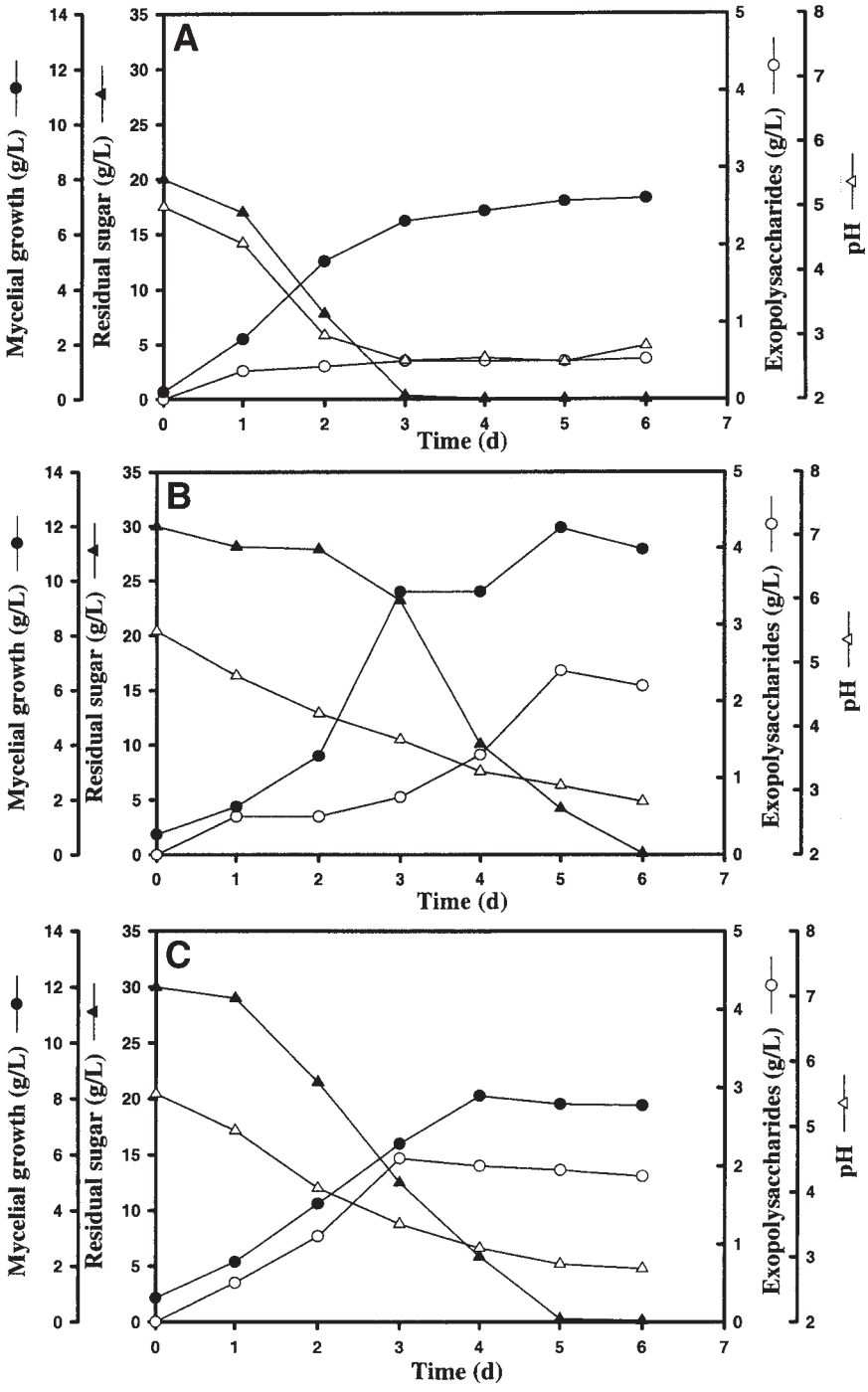

Fig. 3. Typical time courses of mycelial growth and EPS production by *C. maculata* in 5-L stirred-tank reactor **(A)** at basal medium, **(B)** at optimized medium, and **(C)** in a 5-L airlift reactor at optimized medium.

Table 7
Fermentation Kinetics of *C. maculata* TG-1 for Stirred-Tank Reactor and Airlift Reactor Under Two Different Media

Kinetic parameter	Optimum medium		
	Basal medium[a]	Stirred-tank reactor	Airlift reactor
Specific growth rate μ (d^{-1})	0.161	0.230	0.178
Specific consumption rate of substrate, $Q_{S/X}$ (g/[g·d])	0.108	0.249	0.344
Specific production rate of EPS, $P_{P/X}$ (g/[g·d])	0.001	0.020	0.033
Yield of EPS on substrate, $Y_{P/S}$ (g/g)	0.008	0.079	0.097

[a]Data from stirred-tank reactor.

In conclusion, the optimum medium constituents for mycelial growth and EPS production of *C. maculata* were successfully determined in shake-flask cultures using the orthogonal matrix method, which enabled investigation into the influence of controlled factors in a multivariable system. Therefore, this optimization technique can be widely applied to other processes for optimization of submerged culture conditions for mushrooms.

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