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Optimization of submerged culture conditions for mycelial growth and exo-biopolymer production by *Paecilomyces tenuipes* C240

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Abstract

This paper is concerned with optimization of submerged culture conditions for mycelial growth and exo-biopolymer production by *Paecilomyces tenuipes* C240 by one-factor-at-a-time and orthogonal matrix methods. The one-factor-at-a-time method was adopted to investigate the effects of medium components (i.e. carbon, nitrogen, and mineral sources) and environmental factors (i.e. initial pH and temperature) on mycelial growth and exo-biopolymer production. Among these variables, glucose, KNO_3, K_2HPO_4, and $MgSO_4$ were identified to be the most suitable carbon, nitrogen, and mineral sources, respectively. The optimal temperature and initial pH for mycelial growth and exo-biopolymer production were 28 °C and 6.0, respectively. Subsequently, the concentration of glucose, KNO_3, K_2HPO_4, and $MgSO_4$ were optimized using the orthogonal matrix method. The effects of media composition on the mycelial growth of *P. tenuipes* C240 were in the order of glucose > K_2HPO_4 > KNO_3 > $MgSO_4$, and those on exo-biopolymer production were in the order of glucose > K_2HPO_4 > $MgSO_4$ > KNO_3. The optimal concentration for enhanced production were determined as 4 g/l glucose, 0.6 g/l KNO_3, 0.1 g/l K_2HPO_4, and 0.1 g/l $MgSO_4 \cdot 5H_2O$ for mycelial yield, and 3 g/l glucose, 0.4 g/l KNO_3, 0.1 g/l K_2HPO_4, and 0.1 g/l $MgSO_4 \cdot 5H_2O$ for exo-biopolymer production, respectively. The subsequent verification experiments confirmed the validity of the models. This optimization strategy in shake flask culture lead to a mycelial yield of 10.18 g/l, and exo-biopolymer production of 1.89 g/l, respectively, which were considerably higher than those obtained in preliminary studies. Under optimal culture conditions, the maximum exo-biopolymer concentration in a 5 l stirred-tank bioreactor was 2.36 g/l.

Keywords: Exo-biopolymer; Optimization; *Paecilomyces tenuipes* C240; Submerged culture

1. Introduction

Paecilomyces tenuipes is one of the famous Chinese medicinal entomopathogenic fungi together with other fungi such as *Cordyceps sinensis* and *Cordyceps militaris.* Both *Paecilomyces* and *Cordyceps* were genera of the family Clavicipitaceae. The fruit bodies of entomopathogenic fungi are highly valued as medicinal herbs, due to their various biological and pharmacological activities including immuno-stimulating and anti-tumor activities [1–3]. Some bioactive constituents from *P. tenuipes* have been reported [4,5], and artificial cultivation techniques have been developed for these fungi and large-scale production of *P. tenuipes* has become possible. Although many investigators have attempted to obtain optimal submerged cultures for exo-biopolymer production from several fungi [6–9], to the best of our knowledge, the nutritional requirements and environmental conditions for submerged culture of *P. tenuipes* have not been demonstrated.

Medium optimization by the one-factor-at-a-time method involves changing one independent variable (i.e. nutrient, temperature, pH, etc.) while fixing the others at certain levels. This single-dimensional search is laborious and time-consuming, especially for a large number of variables, and frequently does not guarantee the determination of optimal conditions. Hence, as a more practical method, the orthogonal matrix method

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was employed to study the relationships between the medium components and their effects on mycelial growth and exo-biopolymer production.

The purpose of this study was to optimize the submerged culture conditions to produce simultaneously produce mycelial biomass and exo-biopolymer by *P. tenuipes* C240 using a statistically based experimental design. In the first step, the one-factor-at-a-time method was used to investigate effects of variables of medium composition (i.e. carbon, nitrogen, and mineral sources) and environmental factors (i.e. pH and temperature) on mycelial growth and exo-biopolymer production. Subsequently, the concentration of the medium components was optimized using an orthogonal matrix method.

2. Materials and methods

2.1. Microorganism and growth conditions

P. tenuipes C240 was kindly provided by Dr J.M. Sung of Kangwon National University, Chuncheon, South Korea and was used throughout this study. Stock cultures were maintained on potato dextrose agar (PDA) slant. Slants were incubated at 25 °C for 6 days and stored at 4 °C. The seed culture was grown in a 250 ml flask containing 50 ml of YM medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 1% glucose) at 25 °C on a rotary shaker incubator (150 rev/min) for 4 days. Flask culture experiments were performed in 250 ml flasks containing 50 ml media after inoculating with 4% (v/v) of the seed culture.

2.2. Inoculum preparation

P. tenuipes C240 was initially grown on PDA medium in a Petri dish, and transferred into the seed medium by punching out 5 mm of the agar plate culture with a self-designed cutter [10].

2.3. Fermentation in a bioreactor

The fermentation medium was inoculated with 4% (v/v) of the seed culture and then cultivated in a 5 l stirred-tank fermenter (KoBioTech Co., Seoul, South Korea). Unless otherwise specified, fermentations were performed under the following conditions: temperature, 28 °C; aeration rate, 2 vvm; agitation speed, 120 rpm; initial pH, 6.0; working volume, 3 l. All experiments were performed at least in triplicates.

2.4. Analytical methods

Samples collected at various intervals from shake flasks were centrifuged at 9000 × *g* for 15 min, and the resulting supernatant filtered through a membrane filter (0.45 µm, Millipore). The resulting culture filtrate was mixed with four times volume of absolute ethanol, stirred vigorously and kept overnight at 4 °C. The precipitated exo-biopolymer was centrifuged at 9000 × *g* for 15 min discarding the supernatant [11]. The precipitate of pure exo-biopolymer was lyophilized and the weight of the polymer was estimated. The dry weight of mycelium was measured after repeated washing of the mycelial pellet with distilled water and drying at 70 °C for overnight to a constant weight. The filtrate from membrane filtration was analyzed by HPLC (Shimadzu Co., Kyoto, Japan) using an Aminex HPX-42C column (0.78 × 30 cm, Bio-rad Laboratories, Hercules, CA, USA) equipped with a refractive index detector for quantitative analysis of residual sugar concentration [11].

Table 1
Effect of carbon source on the mycelial growth and exo-biopolymer production by *P. tenuipes* C240 in shake flask cultures

Sugar (1%)	Dry cell weight (g/l)	Exo-biopolymer (g/l)	Final pH
Fructose	7.28 ± 0.33	0.57 ± 0.03	4.7
Glucose	8.60 ± 0.46	0.82 ± 0.07	4.5
Lactose	5.36 ± 1.35	0.54 ± 0.06	5.5
Maltose	9.72 ± 2.35	0.36 ± 0.12	4.8
Sucrose	8.52 ± 2.05	0.39 ± 0.01	3.7
Xylose	4.04 ± 1.05	0.39 ± 0.03	4.8

Fermentation were carried out for 5 days at 25 °C with initial pH 5. Values are mean ± S.D. of triple determinations.

Table 2
Effect of nitrogen source on the mycelial growth and exo-biopolymer production by *P. tenuipes* C240 in shake flask cultures

Nitrogen (2%)	Dry cell weight (g/l)	Exo-biopolymer (g/l)	Final pH
Corn steep powder	9.15 ± 1.01	0.39 ± 0.07	5.1
Meat peptone	13.23 ± 0.85	0.51 ± 0.11	5.2
Poly-peptone	11.62 ± 2.04	0.20 ± 0.02	4.6
Tryptone	13.96 ± 0.56	0.16 ± 0.02	4.7
Yeast extract	7.89 ± 1.99	0.28 ± 0.05	4.8
Ammonium phosphate	6.05 ± 0.03	0.31 ± 0.04	4.8
Potassium nitrate	7.38 ± 1.72	0.61 ± 0.12	5.5

Fermentation were carried out for 5 days at 25 °C with initial pH 5. Values are mean ± S.D. of triple determinations.

3. Results and discussion

3.1. One-factor-at-a-time method

3.1.1. Effect of carbon source

To find a suitable carbon source for mycelial growth and exo-biopolymer production in *P. tenuipes* C240,

Table 3
Effect of mineral source on the mycelial growth and exo-biopolymer production by *P. tenuipes* C240 in shake flask cultures

Mineral sources (5 mM)	Dry cell weight (g/l)	Exo-biopolymer (g/l)	Final pH
Control	6.93±0.13	0.61±0.04	6.0
$CaCl_2$	6.52±0.99	0.73±0.04	5.3
$FeSO_4 \cdot 7H_2O$	4.07±1.11	0.48±0.14	6.1
K_2HPO_4	8.74±0.14	0.93±0.03	6.4
$MgSO_4 \cdot 5H_2O$	8.23±0.41	0.85±0.04	6.2
$MnCl_2$	7.54±1.38	0.64±0.18	5.8

Fermentation were carried out for 5 days at 25 °C with initial pH 5. Values are mean±S.D. of triple determinations.

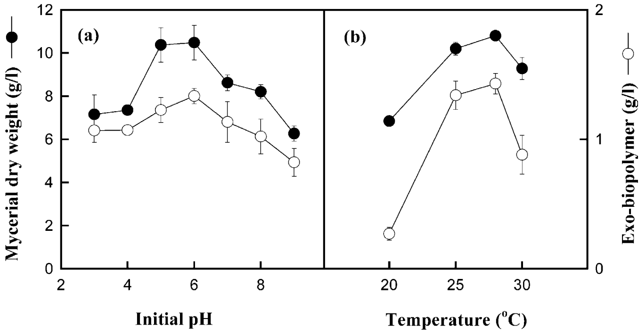

Fig. 1. Effects of initial pH (a) and temperature (b) on mycelial growth (●) and exo-biopolymer production (○) by *P. tenuipes* C240 in shake flask cultures. Experimental data are mean±S.D. of triple determinations.

various carbon sources were provided at a concentration of 10 g/l for 6 days in the basal medium. Among the carbon sources tested, the highest mycelial growth and exo-biopolymer production were obtained in glucose medium Table 1.

3.1.2. Effect of nitrogen source

To investigate the effect of nitrogen sources on mycelial growth and exo-biopolymer production, cells were cultivated in the medium containing various nitrogen sources, where each nitrogen source was added to the basal medium at a concentration level of 2 g/l. Amongst seven kinds of nitrogen sources examined, meat peptone and tryptone were favorable for the mycelial growth of *P. tenuipes* C240 (Table 2). However, maximal exo-biopolymer production was achieved when potassium nitrate was employed. Taking into account that higher fungi usually require long periods for successful submerged cultures exposing these to contamination problem, this inorganic nitrogen source optimum is regarded as a desirable physiological property.

3.1.3. Effect of mineral source

The influence of mineral sources on mycelial growth and exo-biopolymer production was examined by various mineral sources at the concentration level of 5 mM.

Table 4
Experimental factors and their levels for orthogonal projects

Level	Glucose (A)%	KNO_3 (B)%	$MgSO_4 \cdot 5H_2O$ (C)%	K_2HPO_4 (D)%
1	3	0.2	0.05	0.05
2	4	0.4	0.10	0.10
3	5	0.6	0.15	0.15

Symbols A, B, C, and D represent factors of glucose, KNO_3, $MgSO_4 \cdot 5H_2O$, and K_2HPO_4. Symbols 1, 2, and 3 represent concentration levels of each factor.

Among the various mineral sources examined, K_2HPO_4 and $MgSO_4$ yielded good mycelial growth and exo-biopolymer production (Table 3).

3.1.4. Effect of initial pH and temperature

In order to investigate the effect of initial pH on mycelial growth and exo-biopolymer production, *P. tenuipes* C240 was cultivated with different initial pHs (3.0–9.0) in shake flask cultures. The optimal pH for mycelial growth and exo-biopolymer production was 6.0 (Fig. 1a). It has been reported that many kinds of ascomycetes and basidiomycetes have more acidic pH optima during submerged culture [12]. To determine the optimal temperature for mycelial growth and exo-biopolymer production, this organism was cultivated at various temperatures, where the optimum temperature was found to be 28 °C (Fig. 1b).

3.2. Orthogonal matrix method

To investigate the relationships between variables of medium components and optimize their concentrations for mycelial growth and exo-biopolymer production, the orthogonal matrix $L_9(3^4)$ method can be used. To reach the same results as those of the orthogonal matrix method, $3^4 \times 2$ replicates, that is, 162 experiments are necessary to achieve experimental goals for full-factors experimental projects. Orthogonal projects, as a result of the suitable design of factors, can give effective responses. They have been successfully applied to improvement of culture media for the production of

Table 5
Application of $L_9(3^4)$ orthogonal projects to the mycelial growth and exo-biopolymer production by *P. tenuipes* C240

Run	A	B	C	D	Dry cell weight (g/l)	Exo-biopolymer (g/l)
1	1	1	1	1	5.49±0.05	1.17±0.19
2	1	2	2	2	7.45±0.31	1.90±0.05
3	1	3	3	3	7.11±0.35	1.28±0.13
4	2	1	2	3	8.41±0.71	1.15±0.18
5	2	2	3	2	6.31±0.41	0.88±0.02
6	2	3	1	1	9.45±0.21	1.02±0.08
7	3	1	3	2	6.45±0.55	1.03±0.04
8	3	2	1	3	5.46±1.00	0.71±0.09
9	3	3	2	1	6.66±0.62	0.82±0.14

The arrangements of column A, B, C, and D were decided by orthogonal design for 4 (factor) × 9 (run number); every row of run number represents one experimental replicate, every run was replicated twice. Values are mean ± S.D. of triple determinations.

Table 6
Analysis of media on mycelial growth and exo-biopolymer production by *P. tenuipes* C240 in shake flask cultures with orthogonal projects

	Dry cell weight (g/l)				Exo-biopolymer (g/l)			
	A	B	C	D	A	B	C	D
K_1	20.05±0.71[a]	20.35±1.31	20.40±1.26	18.46±0.88	4.35±0.37	3.35±0.51	2.90±0.36	2.88±0.41
K_2	24.17±1.32	19.22±1.72	22.52±2.06	23.35±1.27	3.05±0.28	3.48±0.16	3.94±0.37	3.94±0.11
K_3	18.57±2.17	23.22±1.78	19.87±1.31	20.98±2.06	2.56±0.27	3.13±0.35	3.19±0.19	3.14±0.40
k_1	6.68±0.24[b]	6.78±0.44	6.80±0.42	6.15±0.29	1.45±0.12	1.12±0.17	0.97±0.12	0.96±0.14
k_2	8.06±0.44	6.41±0.57	7.51±0.69	7.78±0.42	1.02±0.09	1.16±0.05	1.31±0.12	1.31±0.04
k_3	6.19±0.72	7.74±0.39	6.62±0.44	6.99±0.69	0.85±0.09	1.04±0.12	1.06±0.06	1.05±0.13
R	1.87[c]±1.16	1.33±0.96	0.88±1.13	1.63±0.71	0.57±0.21	0.12±0.17	3.22±0.24	0.20±0.18
Optimal level	2	3	2	2	1	2	2	2

[a] $K_i^{\mathrm{A}} = \Sigma$ mycelial yield at A_i. Values are mean ± S.D. of triple determinations.
[b] $k_i^{\mathrm{A}} = K_i^{\mathrm{A}}/3$. Values are mean ± S.D. of triple determinations.
[c] $R_i^{\mathrm{A}} = \max\{k_i^{\mathrm{A}}\} - \min\{k_i^{\mathrm{A}}\}$. Values are mean ± S.D. of triple determinations.

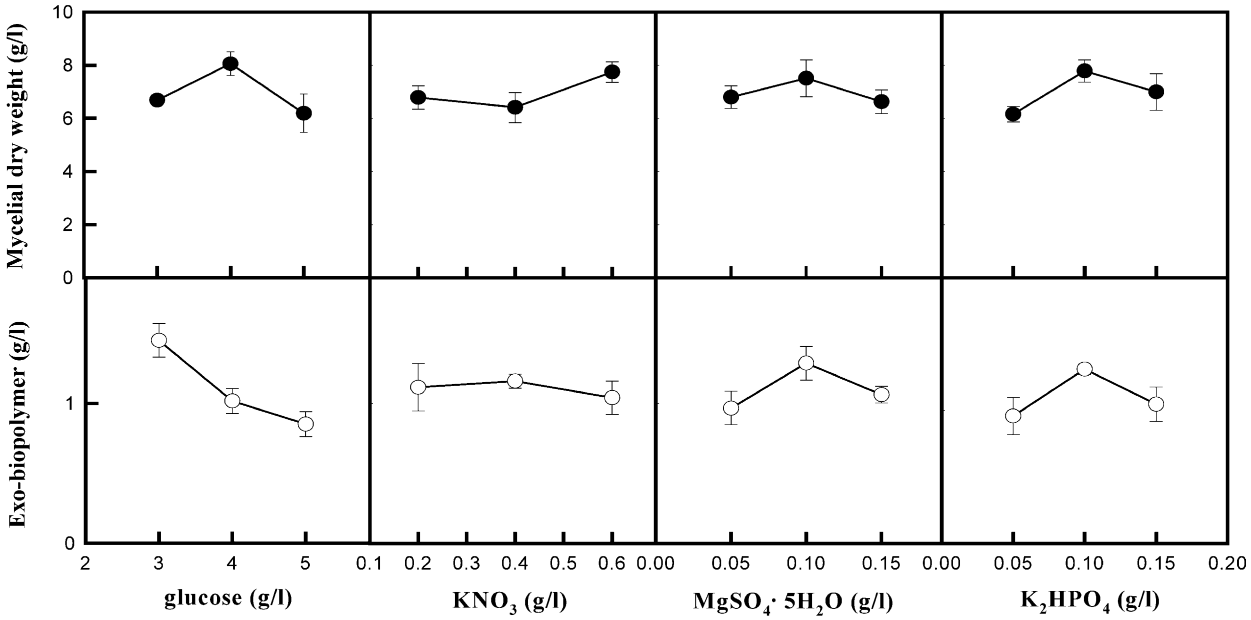

Fig. 2. Intuitive analysis of the relationship between media and mycelial growth (●)/exo-biopolymer production (○) by *P. tenuipes* C240 in shake flask cultures. Experimental data are mean ± S.D. of triple determinations.

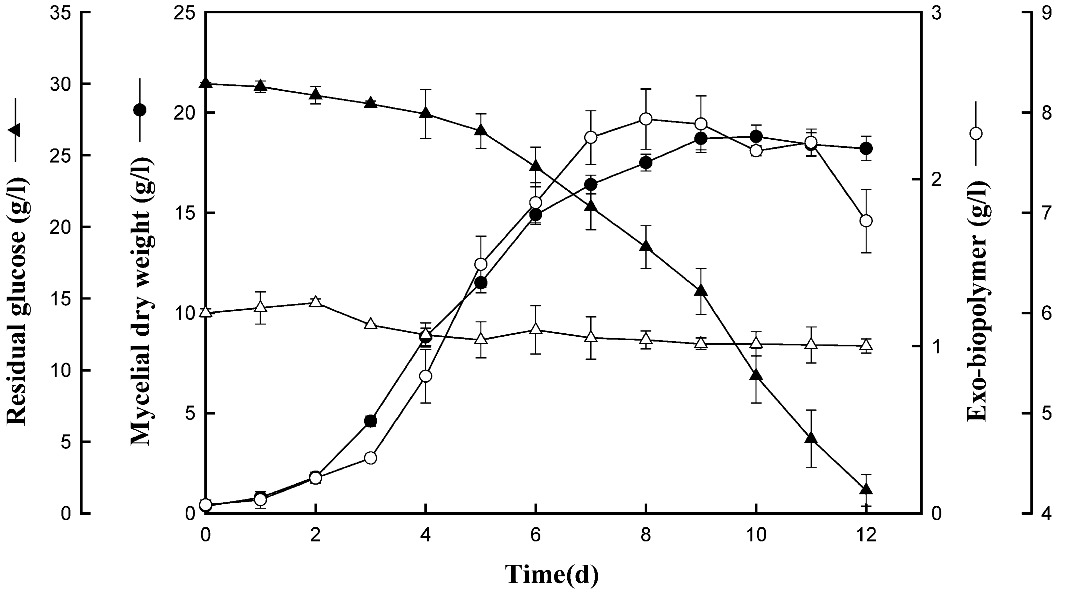

Fig. 3. Typical time courses of the mycelial growth and exo-biopolymer production by *P. tenuipes* C240 in the suggested medium in 5 l stirred-tank bioreactor. Experimental data are mean ± S.D. of triple determinations. (●) Mycelial dry weight, (○) exo-biopolymer, (▲) residual sugar, (△) pH.

primary and secondary metabolites in fermentation process [13–15]. According to preliminary experiments, with only 9×2 replicates ($= 18$) experiments of $L_9(3^4)$ orthogonal projects, we selected and varied three levels as shown in Table 4. The experimental conditions for each project are listed in Table 5, and experimental results are included in the last two columns. The fermentation conditions of temperature, initial pH, agitation rate, and growth period were fixed to be 28 °C, 6.0, 150 rpm, and 5 days, respectively.

3.2.1. *Order of effects of factors*

According to the orthogonal method [15–17], the effect of those media on mycelial growth and exo-biopolymer production was calculated and the results are shown in Table 6. According to the magnitude order of *R* (Max Dif), the order of effect of all factors on mycelial growth could be determined. The order of effects of factors on mycelial growth was glucose > $K_2HPO_4 > KNO_3 > MgSO_4$. Applying the same method, the order of effects of factors on exo-biopolymer production was glucose > $K_2HPO_4 > MgSO_4 > KNO_3$. This result pointed out that the effect of glucose was more important than that of other nutrients.

3.2.2. *Optimum levels of each factor*

To obtain the optimization levels or composition of each factor, the intuitive analysis based on statistical calculation using the data in Table 6, is shown in Fig. 2. The results were as follows: (1) to obtain a high mycelial growth, the optimum composition were 4% glucose, 0.6% KNO_3, 0.1% $MgSO_4 \cdot 5H_2O$, and 0.1% K_2HPO_4; (2) to obtain a high exo-biopolymer production, the optimum composition was 3% glucose, 0.4% KNO_3, 0.1% $MgSO_4 \cdot 5H_2O$, and 0.1% K_2HPO_4.

To confirm these data, experiments were carried out using these nutrient concentrations and 10.18 g/l of mycelial biomass and 1.89 g/l of exo-biopolymer were obtained. This implied that the selected conditions were the most suitable in practice.

3.3. *Fermentation results*

Fig. 3 shows the typical time courses of mycelial growth and exo-biopolymer production in a 5 l stirred-tank bioreactor under optimal culture conditions (3% glucose, 0.4% KNO_3, 0.1% $MgSO_4 \cdot 5H_2O$, and 0.1% K_2HPO_4) for exo-biopolymer production. The maximum exo-biopolymer production indicated 2.36 g/l after 8 days of fermentation, while maximum mycelial yield was 18.8 g/l after 10 days. The variance in pH value during fermentation was not so significant. Optimization of operating parameter (e.g. agitation, aeration, and dissolved oxygen tension) in bioreactor fermentation deserves further investigation, which is being carried out in our laboratory.

4. Conclusions

Using the one-factor-at-a-time method and orthogonal matrix method, it was possible to determine optimal operating conditions to obtain a high exo-biopolymer yield in *P. tenuipes* C240. Two optimization techniques used in this work can be widely applied to other processes for optimization of submerged culture conditions for the mushrooms.

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