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Optimization of medium components and incubation time for the production of *Paecilomyces tenuipes* mycelia in submerged culture

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ABSTRACT: The choice of the culture medium is an important factor for the mass production of mycelia in submerged cultures. The influence of liquid medium on the mycelial dry weight of *Paecilomyces tenuipes* was investigated in this study. The regression equation is expressed as $Y=-1292.94187+17.78612X_1+18.92425X_2+2.11464X_3-0.019375X_1X_2-0.006276X_1X_3+0.008177X_2X_3-0.070169X_1^2-0.292175X_2^2-0.008818X_3^2$, where Y represents the value of the mycelial dry weight (g/L), X_1 is the particle size of wood sawdust in liquid medium (mesh), X_2 is the concentration of the wood sawdust in liquid medium, and X_3 is incubation time (h). The medium was optimized using a response surface methodology, and the optimal medium contained 30 g of wood sawdust (140 mesh), 20 g of glucose, and 10 g/L of peptone. Under these conditions, the mycelial dry weight reached 38.1 g/L (actual value). The culture medium containing wood sawdust is simple and easy to use, highly efficient, and eco-friendly, and its effectiveness in large preparations of *P. tenuipes* mycelia with low material costs has been demonstrated.

KEYWORDS: Medium, Mycelium, *Paecilomyces tenuipes*, Response surface methodology, Submerged culture

INTRODUCTION

Cordyceps, an ascomycete, is one of the most valued traditional Chinese medicines (Dong and Yao, 2005). A caterpillar-shaped Chinese traditional medicinal mushroom, it is an entomopathogenic fungus that belongs to the class Ascomycetes and Dong Chong Xia Cao group of Chinese herbs (Mao *et al*., 2005). Previous studies have demonstrated its multiple pharmacological activities, including anti-inflammatory (Yang *et al*., 2010), analgesic (Paterson, 2008), stimulation of steroidogenesis (Ikeda *et al*., 2008), immunity enhancement (Wang *et al*., 2012), and antitumor activities (Pao *et al*., 2012). The fungus is endemic to the Tibetan Plateau and may be found only above 3000 m up to the snow line, usually between 3600 and 5000 m (Li *et al*., 2019).

Natural production of the fungus is limited, and the annual yield has been declining continually over recent years (Yang, 1997). The fall in supply and increase in demand have stimulated interest in the search for substitutes for formulating natural products of the fungus. It has been observed that the mycelial fermentation products have the same or stronger pharmacological efficacy than the wild *Cordyceps*. However, a cultivation method for the production of fruiting bodies has not yet been developed successfully. Hence, the production of mycelia by submerged fermentation is being viewed as a promising alternative (Li *et al*., 2001). Growth in submerged cultures has the potential advantage of high mycelial mass production under specified manufacturing conditions (Dong and Yao, 2005). Submerged cultures have many potential advantages, such as production of higher mycelial biomass in a compact space and shorter time with less chances of contamination (Lee *et al*., 2004). Although

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the nutritional requirements of some species of *Cordyceps* (Fr.) Link grown in submerged culture have been determined (Dong and Yao, 2005; Shih *et al*., 2007; Cui and Zhang, 2011), fewer studies have been conducted with regards to *Paecilomyces tenuipes*. Xiao *et al*. (2006) reported a liquid medium containing 40 g/L of molasses glycerol, 5 g/L of peanut steep meal, 6 g/L of yeast extract, 1 g/L of KH_2PO_4, 0.5 g/L of $ZnCl_2$, 0.5 g/L of $MgSO_4$ at an initial pH of 6 to be optimal for the mycelial growth of *Cordyceps* (24.5 g/L).

Pokhrel and Ohga (2007) reported that yeast extract was the best nitrogen source for the production of *Cordyceps* mycelia (7.03 g/L). Xu *et al*. (2003) tested different media compositions for the mycelial growth of *P. tenuipes* C240 and found that the media promoted growth in the following order: glucose> K_2HPO_4> KNO_3>$MgSO_4$. Bae *et al*. (2001) reported that the maximum mycelial concentration of *P. japonica* achieved in sucrose medium (25 g/L) was higher than that achieved in maltose medium (20 g/L). Compared with other *Cordyceps* species, the nutritional requirements of *P. tenuipes* are less well known. Further information is required for cultivating this species through fermentation. The first step is to promote growth and increase biomass in submerged culture.

In this study, the effect of different liquid medium components on mycelia production of *P. tenuipes* in submerged cultures was evaluated. In addition, this study aimed to optimize culture medium components and incubation time to obtain maximal production of mycelia of *P. tenuipes*. The information obtained would be beneficial not only for large-scale production of mycelia but also for further manipulation and understanding of cordycepin biosynthesis in the future.

MATERIALS AND METHODS

Maintenance and seed culture

The *P. tenuipes* strain was purchased from the Korean Culture Center of Microorganisms (Seoul, South Korea). The stock culture was maintained on potato-dextrose-agar plates. The plates were inoculated with mycelia and incubated at 24°C for 14 days, and then used for seed culture inoculation. The seed culture medium consisted of 20 g/L glucose and 10 g/L peptone, adjusted to a pH of 4.5. The mycelia of *P. tenuipes* were transferred to the seed culture medium by punching wells of about 5 mm^2 of the plates with a sterilized punching machine. The seed culture was grown in a 250 mL shake flask containing 50 mL of liquid medium and incubated at 24°C on a rotary shaker (100 rpm) for five days.

Preparation of liquid medium and components

In order to investigate the effect of various particle sizes and concentrations of wood sawdust on the dry weight enhancement of mycelia of *P. tenuipes* in the submerged medium, 5–30 g of wood sawdust (*Quercus variabilis* Blume) with particle sizes ranging from 100–140 mesh (0.15-0.105 mm) was added to per L of the medium (pH 4.5; glucose 20 g/L; peptone 10 g/L). The culture flasks were incubated at 24°C and 100 rpm for five days. In all experiments, multiple flasks, at least in duplicate, were run at the same time to ensure reproducibility. All incubations were performed five times, and these results were used as data for the response surface methodology (RSM) analysis.

Determination of mycelial dry weight

For the measurement of mycelial dry weight, the cells from a sample were filtered through a filter paper and washed twice with distilled water. The fresh mycelia were dried at 60°C for sufficient time until a constant mycelial dry weight was obtained. The mycelial dry weight was calculated by subtracting the dry weight of the wood sawdust from these dried samples. After sampling, one part of the culture filtrate was used for measuring cordycepin content value using high performance liquid chromatography (HPLC).

Experimental design by Box-Behnken design (BBD) of response surface methodology (RSM) for optimization of liquid medium composition and incubation time

The Box–Behnken design (BBD) was used to determine the optimum level of the most significant variables (wood sawdust particle size, X_1; wood sawdust concentration, X_2; incubation time, X_3) and study their interactions. Each effective variable in the design was studied at three different levels (coded as -1, 0, and +1), as shown in Table 1. A total of 17 experiments were carried out, and the entire experimental design considered three center points. The experimental results were fitted with a multiple regression analysis, explained by a quadratic polynomial equation (Eq. (1)):

$$Y = \beta_0 + \Sigma_{i=1}^{4}\beta_i x_i + \Sigma_{i=1}^{4}\beta_{ii} x_i^2 + \Sigma_{i=1}^{3}\Sigma_{i<j}^{4}\beta_{ij} x_i x_{ij} \quad \text{Eq. (1)}$$

where Y is the predicted response (mycelial dry weight in

this study, mg/mL), β0, βi, βii, and βij are constant coefficients, and x_i and x_j are the coded independent variables or factors.

The fitness of the second-order polynomial model was expressed via the regression coefficient R^2, and a detailed analysis of variance (ANOVA) was conducted at a coded level of variables to determine the effects of individual variables.

SAS software (version 11.0, SAS Institute Inc., Cary, NC, USA) was used for the regression analysis and graphical analysis of the experimental data.

Determination of cordycepin content by high performance liquid chromatography

An HPLC system (Dalian Elite Analytical Instrument Ltd., Dailian, China) with a vacuum degasser, quaternary pump, UV/Vis detector, and analytical software was used for the detection and analysis of cordycepin. The HPLC conditions were as follows: column, Agilent Eclipse plus C18 (250 mm × 4.6 mm, 5 μm); mobile phase, methanol: water (20:80, v/v); flow rate, 1.0 mL/min; UV detection at 260 nm; and injection volume, 10 μL. The samples were filtered through a 0.45 μm membrane filter before injection. Cordycepin was quantitively analyzed by the peak area based on their standard curves. Peaks for cordycepin in the samples were identified by their retention times.

Statistical analysis

Data are presented as the mean standard deviation (n = 3). Statistical analyses of the results were performed at a 5% significance level using the Statistical Analysis System software (SAS Institute, Inc., 2000). Differences between the means of individual groups were assessed using SAS with Duncan's multiple-range test.

RESULTS AND DISCUSSION

Effect of different liquid media on mycelial dry weight

Carbohydrate raw material is an important carbon and energy source for cultured cells (Silva *et al*., 2017; Tang *et al*., 2017). The effects of various carbohydrate raw materials on mycelial growth of *Cordyceps* have been reported (Park *et al*., 2001), but less research has been conducted on wood sawdust-based liquid medium for mycelial growth of the strain. To identify a suitable raw material for submerged cultivation of *P. tenuipes*, an oak wood sawdust-based liquid medium was used. Other carbohydrate raw material sources, including sawdust of pine wood, Japanese cedar wood, and tulip tree wood, were also tested in preliminary experiments, but a low mycelial dry weight was obtained (data not shown). The mycelial dry weights under liquid medium supplemented with various particle sizes of wood sawdust are compared in Fig. 1. Henceforth, the term wood sawdust refers to oak wood sawdust.

After the initial experiments, the amount of wood sawdust added to the liquid medium was fixed at 20 g/L. Smaller the particle size of wood sawdust contained in the liquid medium, higher was the mycelial dry weight. Significant mycelial dry weight was obtained with liquid medium containing wood sawdust at all

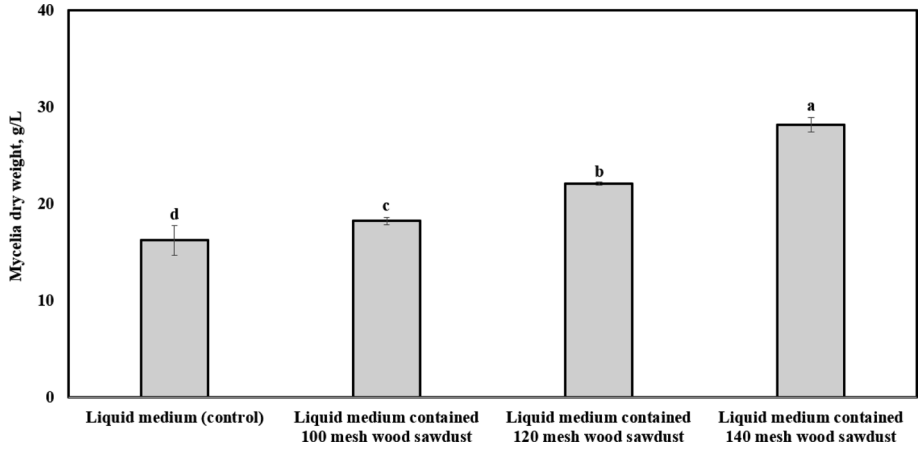

Fig. 1. Effect of particle size of wood sawdust on the mycelia dry weight in liquid medium during submerged culture of *P. tenuipes*. Liquid medium (control) was used pH, 4.5; glucose 20 g/L; peptone 10 g/L; without wood sawdust. Wood sawdust input amount: 20 g/L. Incubation time: 5 days. The error bars in the figure indicate the standard derivations from five independent samples. Different letters on the top of the line represent statistically significant at 5% probability level.

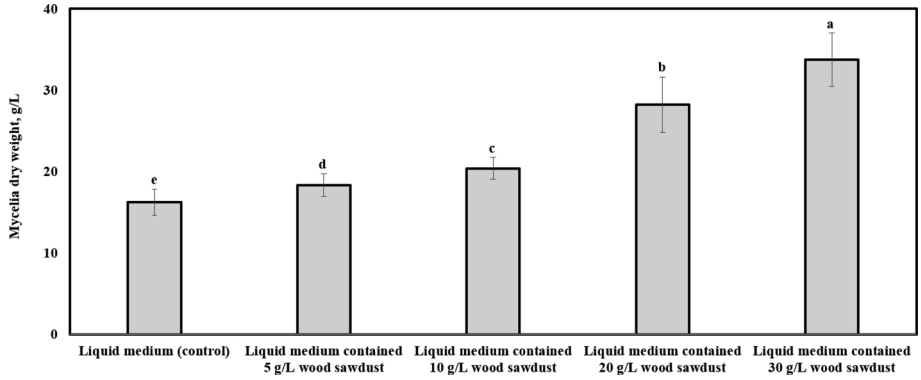

Fig. 2. Effect of amount of wood sawdust on the mycelia dry weight in liquid medium during submerged culture of *P. tenuipes*. Liquid medium (control) was used pH, 4.5; glucose 20 g/L; peptone 10 g/L; without wood sawdust. Wood sawdust particle size: 140 mesh pass (0.105 mm). Incubation time: 5 days. The error bars in the figure indicate the standard derivations from five independent samples. Different letters on the top of the line represent statistically significant at 5% probability level.

particle sizes. However, maximal mycelial dry weight of 28.17 g/L was obtained in liquid medium containing 140 mesh pass (particle size: 0.105 mm) wood sawdust after five days of cultivation while the cordycepin content was 740.13 μg/mL. It is possible that different liquid media may have different effects of catabolic repression on cellular secondary metabolism (Mao *et al*., 2005).

Based on the above results, a liquid medium containing 140 mesh pass wood sawdust was selected as the liquid culture medium for further studies. Fig. 2 shows the effects of liquid medium containing various concentrations of wood sawdust on mycelial dry weight in submerged cultivations of *P. tenuipes*. All the liquid medium containing wood sawdust well supported mycelial growth, with the best results being obtained at 30 g/L wood sawdust. The dry weight of the mycelia obtained from this submerged culture system reached 33.76 g/L while the cordycepin content reached 133.76 μg/mL.

When the concentration of wood sawdust contained in the liquid medium was controlled, the mycelial dry weight increased significantly, but the cordycepin content decreased compared to the particle size control experiment. It was confirmed that the mycelial dry weight and cordycepin content did not show the same trend, and this has also been reported in previous studies (Fan *et al*., 2012). Therefore, there is a need for further research targeting the increase in cordycepin content. The time profiles of mycelial dry weight in liquid medium containing wood sawdust (140 mesh pass (0.105 mm), 30 g/L) are presented in Fig. 3. Maximal mycelial dry weight (35.3 g/L) and growth rate (0.4 g/L/h) were obtained after 96 h of cultivation. The liquid

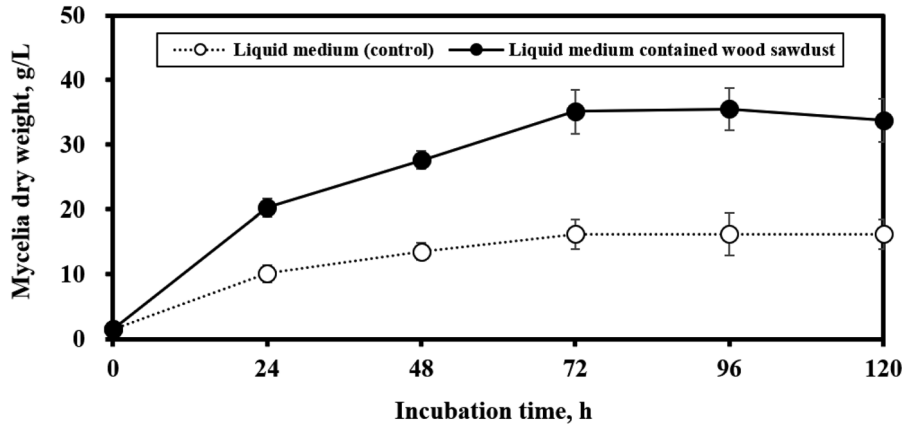

Fig. 3. The time profiles of mycelia dry weight under liquid medium contained wood sawdust (140 mesh pass, 30 g/L). Liquid medium (control) was used pH, 4.5; glucose 20 g/L; peptone 10 g/L; without wood sawdust. The error bars in the figure indicate the standard derivations from five independent samples.

Table 1. Experimental codes, range, and levels of the variables used for the Box-Behnken design (BBD)

Variables [a]	Units	Symbol code	Levels		
			-1	0	+1
Particle size of wood sawdust	Mesh	X_1	100	120	140
Amount of wood sawdust	g/L	X_2	20	30	40
Incubation time	hours	X_3	24	72	120

[a] Based liquid medium: pH, 4.5; glucose 20 g/L; peptone 10 g/L

medium containing wood sawdust (140 mesh pass (0.105 mm), 30 g/L) showed higher mycelial dry weight at all incubation times than the liquid medium containing wood sawdust.

Optimization of screened variables using RSM (BBD) for mycelia growth

We performed RSM analysis to obtain the maximum mycelial dry weight of *P. tenuipes* using the above results (Table 1). The BBD was used to optimize the levels of significant variables (particle size of wood sawdust in liquid medium, concentration of wood sawdust in liquid medium, and incubation time). The actual values for the 17 BBD experiments are shown in Table 2. The second-order polynomial equation provided a mathematical model to describe the relationship between the variables and the response. The regression equation is expressed as follows:

$$Y = -1292.94187+17.78612X_1+18.92425X_2+2.11464X_3 -0.019375X_1X_2-0.006276X_1X_3+0.008177X_2X_3 -0.070169X_1^2-0.292175X_2^2-0.008818X_3^2$$

where Y represents the value of mycelial dry weight (g/L), X_1 is the particle size of wood sawdust in liquid medium (mesh), X_2 is concentration of wood sawdust in liquid medium, and X_3 is incubation time (h). The ANOVA of the optimization indicated the response surface model terms, which are listed in Table 3. The coefficient of determination (R^2) for the model was 0.9772, which could explain the 97.72% variability in the data of the model.

The F-value of the model was 16.79, and the *p* value was 0.0006, which indicated that the experimental data fitted well with the quadratic model. Three-dimensional response surface plots and the corresponding contour plots were used to optimize the levels of all variables for the value of mycelial dry weight (Fig. 4).

From the response surface plots and contour plots, the

Table 2. Box–Behnken experimental design of medium contained pretreated sawdust for cordycepin content of *P. tenuipes* on submerged culture

Run	Code factor value			Mycelia dry weight, Y
	Particle size of wood sawdust, X_1	Amount of wood sawdust, X_2	Incubation time, X_3	
1	0	-1	-1	7.75
2	0	0	0	13.18
3	0	0	0	14.38
4	-1	0	1	11.87
5	-1	1	0	7.36
6	0	0	0	12.56
7	1	0	-1	6.85
8	0	0	0	13.37
9	1	-1	0	9.15
10	-1	0	-1	6.82
11	0	0	0	14.49
12	0	-1	1	9.59
13	0	1	-1	6.91
14	1	1	0	7.06
15	1	0	1	9.49
16	0	1	1	10.32
17	-1	-1	0	7.90

Table 3. ANOVA results of the fit model from Box-Behnken design

Source	Sum of squares	df	Mean Square	F-value	p-value	
Model	12112.24	9	1345.80	16.79	0.0006	significant
A-A	24.50	1	24.50	0.3057	0.5976	
B-B	93.84	1	93.84	1.17	0.3151	
C-C	2093.05	1	2093.05	26.11	0.0014	
AB	60.06	1	60.06	0.7494	0.4154	
AC	145.20	1	145.20	1.81	0.2203	
BC	61.62	1	61.62	0.7688	0.4097	
A^2	3316.99	1	3316.99	41.38	0.0004	
B^2	3594.37	1	3594.37	44.84	0.0003	
C^2	1738.11	1	1738.11	21.69	0.0023	
Residual	19.64	7	2.81			
Lack of fit	18.79	3	6.26	29.41	0.0035	significant
Pure error	0.8520	4	0.2130			
Cor total	620.97	16				

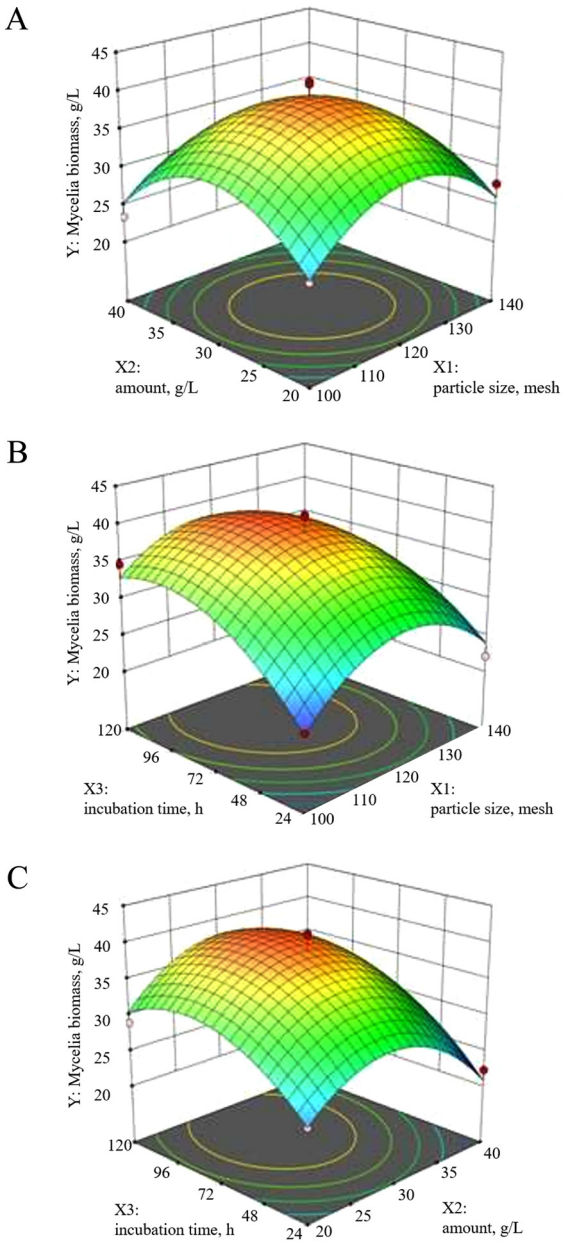

Fig. 4. Response surface graphs for mycelia dry weight *P. tenuipes* as a function of significant variables (particle size of wood sawdust in liquid medium, amount of wood sawdust in liquid medium and incubation time). A: response surface graphs for mycelia dry weight as a function of particle size of wood sawdust in liquid medium and amount of wood sawdust in liquid medium; B: response surface graphs for mycelia dry weight as a function of particle size of wood sawdust in liquid medium and incubation time; C: response surface graphs for mycelia dry weight as a function of amount of wood sawdust in liquid medium and incubation time.

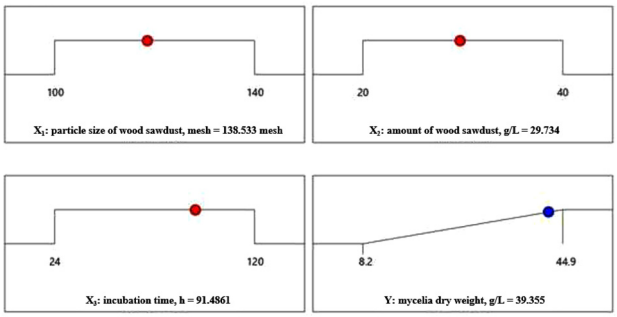

Fig. 5. Optimization of mycelia dry weight *P. tenuipes* as a function of significant variables by Response surface graphs.

value of mycelial dry weight was found to present a maximum in the tested range. Based on the aforementioned equation and the response surface plots, the optimum levels of the three variables were as follows: the particle size and concentration of wood sawdust in liquid medium was 138.533 mesh and 29.734 g/L respectively, and incubation time was 91.4861 h (Fig. 5). The maximum value of mycelial dry weight, which could be obtained by the model, was 39.4 g/L (Table 4). The final optimized medium contained 30 g/L wood sawdust (140 mesh), glucose 20 g/L, and peptone 10 g/L. In order to validate the predicted results of the statistical model, experiments using the optimized medium composition were conducted that resulted in mycelial dry weight of 38.1 g/L. This confirms the validity of the model in simulating and predicting the value of mycelial dry weight. Whether a specific compound of wood sawdust is beneficial to mycelial growth of *P. tenuipes* is currently under investigation in this laboratory. Submerged cultivation of *Cordyceps* is a promising alternative to wild cultivation and solid-state fermentation (Suparmin *et al*., 2017; Jiang *et al*., 2018).

Our research also proved that submerged cultivation of *P. tenuipes* under the present cultivation conditions can achieve high amounts of mycelial dry weight. Till date, relatively low levels of mycelial dry weight have been produced in submerged cultivation of *Cordyceps* species. Mycelial dry weight of 21.3 mg/L was reported in submerged cultivation in liquid medium containing yeast extract (Mao and Zhong, 2006). A submerged culture method for mycelia production of *Cordyceps* on various liquid medium conditions was developed by Xie *et al*., (2009) that remarkably improved the production efficiency, and highest mycelial dry weight of 19.1 g/L was obtained. To further enhance the mycelial dry weight by submerged cultivation of *Cordyceps*, the effects of 5 L and 100 L fermentation were investigated,

Table 4. Results of the verified experiment

	Particle size of wood sawdust, mesh	Amount of wood sawdust, g/L	Incubation time, h	Mycelia dry weight, g/L		Cordycepin content, μg/mL
				Actual	Predicted	
Optimal culture condition	140.0	29.7	91.0	38.1	39.4	121.1

resulting in high mycelial dry weight (17.1 g/L) (Chang *et al*., 2005). Yang *et al*. (2010) achieved 12.2 g/L of mycelial dry weight in a submerged culture containing yeast extract and sucrose. Bae *et al*. (2001) reported that the maximum mycelial concentration of *Paecilomyces japonica* achieved in sucrose medium (25 g/L). Lee *et al*. (2013) reported that the under their optimal conditions, the maximum mycelial growth was 23.1 g/L. The maximum production (38.1 g/L, actual value) of mycelial dry weight obtained in the present study is significantly higher than that reported previously. The results of this study suggest that a liquid medium containing wood sawdust is the most beneficial for enhancing mycelial dry weight in *P. tenuipes* in submerged culture. However, the identification of mechanisms regulating the growth pathways of mycelia deserves further investigation.

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