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Effects of culture conditions on the adenosine production in submerged culture of *Paecilomyces tenuipes*

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ABSTRACT: We studied the effects of initial pH, different nitrogen sources, and cultivation methods (shake flask and static culture) on biomass production, exopolysaccharides (EPS), and adenosine by *Paecilomyces tenuipes*. Relatively low pH levels were optimal for mycelial growth and EPS production. Yeast extract was the most effective organic nitrogen source for EPS production, whereas soybean extract was the best for adenosine production. A high C/N ratio was beneficial for adenosine production; however, excessively high C/N ratios reduced adenosine production. Static fermentation significantly increased adenosine production. A Box-Behnken design was used to optimize adenosine production; the optimal conditions for adenosine production by *P. tenuipes* were pH 7.0, soybean concentration of 3%, and a static culture period of 20 days, with the maximum adenosine production of 141.10 mg/L (predicted value: 128.05 mg/L).

KEYWORDS: Adenosine, growth condition, optimization, *P. tenuipes*

INTRODUCTION

Paecilomyces tenuipes, an entomopathogenic fungus belonging to the Ascomycetes class, is recognized as a medicinal mushroom in Chinese traditional medicine (Xu et al., 2006). Various species of Cordyceps have been used for their medicinal properties in China, Japan, Korea, and other oriental countries, due to their biological and pharmacological activities. These activities are largely due to bioactive compounds such as adenosine, cordycepin, and exopolysaccharides (Shashidhar et al., 2013).

Adenosine is acknowledged as a promising cardio-protective and therapeutic agent for chronic heart failure. Polysaccharides are known for their properties including anti-inflammatory, antioxidant, antitumor, antimetastatic, immunomodulatory, hypoglycemic, steroidogenic, and hypolipidemic effects (Quintana et al., 2004; Kumar et al., 2023). Despite adenosine's numerous benefits, most research has primarily concentrated on cordycepin. Adenosine, well-known for its cardioprotective effects, is a bioactive compound found in many medicinal mushrooms, including *Cordyceps* species (Sharma et al., 2023). It interacts with specific G-protein-coupled receptors and various effector systems. Studies indicate that both endogenous and exogenous adenosine play a role in protecting the heart from myocardial ischemia by maintaining adequate blood flow and oxygen supply (de Jong et al., 2000). Furthermore, adenosine offers protective effects against lung ischemia-reperfusion injuries by inhibiting the proinflammatory cytokine IL-6 and increasing the anti-inflammatory cytokine IL-10 (Bouma et al., 1997).

Wild Cordyceps fruiting bodies are expensive due to their host specificity and scarcity in nature; they grow very slowly, are limited to specific regions, and are small in size (Shrestha et al., 2012). Consequently, collecting enough quantities for extensive medicinal use is challenging. Solid-state mushroom culture is time-consuming for fruiting body production, leading to efforts to obtain useful cellular or extracellular substances from submerged mycelial culture for nutraceuticals and functional foods (Rathore et al., 2019). Submerged culture offers several

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advantages, including higher mycelial production in a compact space, shorter cultivation time, and a reduced risk of contamination (Bakratsas et al., 2021).

Although significant research has been conducted on submerged cultivation of mushrooms for valuable metabolite production, the submerged cultivation of *P. tenuipes* has been scarcely studied until recently. This study explored the nutritional requirements and culture conditions for the submerged cultivation of *P. tenuipes* to optimize the production of mycelia, polysaccharides, and adenosine. Additionally, a statistical experimental design approach was used to optimize cultural conditions for adenosine production by *P. tenuipes*. This approach is increasingly employed in various phases of fermentation optimization, as it is an effective technique for testing multiple process variables with fewer experimental trials compared to the traditional on "e-factor-at-a-time" method (Kasemiire et al., 2021). Furthermore, interactions between variables can be identified and quantified using this method. The application of statistical experimental design for optimizing adenosine production by *P. tenuipes* is limited.

Therefore, the findings of this study will provide crucial data that will enable a shift from cordycepin-focused research to a greater emphasis on adenosine.

MATERIALS AND METHODS

Mycelium and seed culture

The *Paecilomyces tenuipes* strain (KACC 40503) was procured from the Rural Development Administration National Institute of Agricultural Sciences. The microorganism was preserved on potato dextrose agar (PDA) slants and sub-cultured on a monthly basis. The mycelium was incubated at 25°C for 7 days and subsequently stored at 4°C. The seed culture medium comprised the following components: 40 g/L glucose, 10 g/L soybean powder, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, and 0.1 g/L $FeSO_4 \cdot 7H_2O$. Agar discs, each approximately 5 mm in diameter, were punched from PDA plates and used to transfer the *P. tenuipes* mycelia to the seed culture medium. Three discs were used to inoculate 100 mL of liquid media. The seed culture was incubated in a 250 mL Erlenmeyer flask at 25°C on a rotary shaker set to 150 rpm for 7 days.

Flask culture conditions

The fermentation medium consisted of 40 g/L glucose, 10 g/L soybean powder, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, and 0.1 g/L $FeSO_4 \cdot 7H_2O$. The initial pH was adjusted to 6 before autoclaving. Flask culture experiments were carried out in 250 mL flasks containing 100 mL of the medium, inoculated with 5% (v/v) of the seed culture. The cultures were incubated at 25°C on a rotary shaker at 150 rpm, and samples were taken at different intervals to analyze biomass dry weight, exopolysaccharides (EPS), and adenosine production.

The effects of various factors on cell growth, EPS, and adenosine production by *P. tenuipes* were investigated using shake flask cultures. Nitrogen source effects were examined by replacing different nitrogen sources, such as peptone, yeast extract, and soybean powder, individually. The impact of initial pH values (ranging from 4.0 to 7.0) on *P. tenuipes* culture was also studied. All experiments were conducted in at least duplicate, and the results were averaged.

Determination of mycelia dry weight, pH and residual sugar

Samples taken at various intervals from the shake flasks were centrifuged at 6000 × g for 10 minutes, and the supernatant was filtered through a pre-weighed Whatman No. 2 filter paper (Whatman International Ltd., Maidstone, UK). The culture filtrate was then used to measure pH and analyze adenosine as described below. The centrifuged mycelia were thoroughly washed with a large amount of distilled water, collected by filtration through the same Whatman filter paper, and freeze-dried to a constant dry weight.

Measurements of exopolysaccharide

The culture filtrate obtained as described above was mixed with four volumes of 95% (v/v) ethanol, stirred vigorously, and left overnight at 4°C. The precipitated exopolysaccharide (EPS) was recovered by centrifugation at 10,290 × g for 10 minutes, and the supernatant was discarded. The crude EPS precipitate was then lyophilized.

Measurement of adenosine by HPLC

Accurate amounts of adenosine (Sigma, USA) were dissolved in a mobile phase solution to prepare various concentrations for calibration. For the analysis of adenosine, the culture filtrate obtained as described above was mixed with 15% methanol (1:1, v/v), followed by centrifugation at 6000 × g for 15 minutes. The supernatant was filtered through a 0.45 μm membrane, and the filtrate was analyzed by HPLC. The HPLC system consisted of a Hitachi L-6200 solvent delivery controller, a RHEODYNE M-4250 injector, a Hitachi 4250 UV–vis detector, a Hitachi D-2500 Chromato-integrator, and a reverse phase C18 column (ACE, UK, 250 mm × 4.6

Table 1. The effects of initial pH on the maximal cell growth and the production of various metabolites by *P. tenuipes*

Cell growth and metabolites	Initial pH in the medium			
	4	5	6	7
Mycelia dry weight (g/L)	21.3±0.2	15.6±0.1	14.9±0.5	14.1±0.7
EPS production (g/L)	1.8±0.0	1.7±0.0	1.7±0.0	1.7±0.1
EPS productivity (g/d)	0.2±0.0	0.2±0.0	0.2±0.0	0.1±0.0
Adenosine production (mg/L)	42.3±0.1	50.2±0.7	78.4±1.2	120.5±0.5
Adenosine productivity (mg/d)	7.8±0.1	12.1±0.2	15.1±0.2	20.7±0.2

Values are the Mean ± SD, and the data shown are average duplicate values. The culture time indicated in the parenthesis was when the maximum cell mass was reached (30 days).

mm, 5 μm particle size). The mobile phase was a mixture of methanol and 0.02 M potassium dihydrogen phosphate (15:85). The injection volume was 20 μL, the column temperature was set at 40°C, the flow rate was 1 mL/min, and the eluent was monitored at 254 nm.

Response surface methodology (RSM), the Box–Behnken design

Box–Behnken design (BBD) was employed to optimize cordycepin production by *P. tenuipes*. This design is a response surface methodology suitable for three or more factors; it is rotatable (or nearly rotatable) and requires only three levels (coded as −1, 0, and +1) for each factor. This method is ideal for exploring quadratic response surfaces and constructing second-order polynomial models. It provides designs with desirable statistical properties while requiring only a fraction of the experiments needed for a full three-level factorial design. For a three-factor, three-level design, the experimental trials consisted of points at the midpoint of each edge of a multidimensional cube and three replications of center points, totaling 17 experiments. The matrix corresponding to the BBD is shown in Table 5, along with the observed experimental data. The BBD experimental results were fitted with a second-order polynomial equation (Eq. (1)) using multiple regression techniques:

$$Y = \beta 0 + \Sigma_{i=1}^{4} \beta i x i + \Sigma_{i=1}^{4} \beta_{ii} x_i^2 + \Sigma_{i=1}^{3} \Sigma_{i<j}^{4} \beta_{ij} x i x j \qquad \text{Eq. (1)}$$

Y is the predicted response (adenosine yield in this study, mg/L), β0, βi, βii, βij are constant coefficients, and xi, xj are the coded independent variables or factors. The quality of fit of the second-order model equation was expressed by the coefficient of determination R^2, and its statistical significance was determined using an F-test. The significance of the regression coefficients was tested using a t-test. The software used for analysis was Statistical (version 5.0) by Statsoft, Inc. (Tulsa, OK, USA).

Table 2. The effects of various nitrogen sources on cell growth and the production of various metabolites by *P. tenuipes*

Cell growth and metabolites	Nitrogen sources		
	Peptone	Yeast extract	Soybean
Mycelia dry weight (g/L)	11.5±0.2	26.1±0.7	15.8±0.1
EPS production (g/L)	0.3±0.0	2.6±0.1	1.7±0.0
EPS productivity (g/d)	0.15±0.0	0.66±0.0	0.40±0.0
Adenosine production (mg/L)	47.8±1.7	41.1±2.3	61.7±0.5
Adenosine productivity (mg/d)	13.5±0.1	13.2±0.1	17.4±0.1

Values are the Mean ± SD, and the data shown are average duplicate values. The culture time indicated in the parenthesis was when the maximum cell mass was reached (30 days).

Statistical Analyses

The data are presented as the mean ± standard deviation (n = 3). Statistical analyses were performed at a significance level of 5% using the Statistical Analysis System software (SAS Institute, Inc., Cary, NC, USA). Differences among group means were assessed using Duncan's multiple-range test with SAS.

RESULTS AND DISCUSSION

Effect of pH on cell growth and the production of metabolites

It has been reported that acidic pH was more suitable for mycelial growth and production of metabolites for many kinds of ascomycetes and basidiomycetes, including Cordyceps sp.,. The effects of initial pH on the maximal production of

Table 3. The effects of yeast extract concentration at various pH on the cell growth and the production of various metabolites by *P. tenuipes*

Cell growth and metabolites	Soybean concentration (g/L) (at initial various pH)				
	10(6)	15(6)	30(6)	30(7)	30(8)
Mycelia dry weight (g/L)	15.8±0.1	11.1±0.2	6.6±0.0	8.9±0.2	7.2±0.0
Adenosine production (mg/L)	61.7±0.5	79.5±0.7	65.3±0.7	100.8±1.5	87.6±1.7

Values are the Mean ± SD, and the data shown are average duplicate values. The culture time indicated in the parenthesis was when the maximum cell mass was reached (30 days).

mycelial biomass and various metabolites by *P. tenuipes* are shown in Table 1. It is seen that the maximal mycelial concentration for pH 4, 5, 6, and 7 was 21.3, 15.6, 14.9, and 14.1 g/L obtained on day 30, respectively (Table 1). These suggest that the cell growth might be due to the high consumption of carbon sources, which led to the production of organic acid and consequently reduced the pH in the media. The maximal EPS production was 1.8, 1.7, 1.7, and 1.7 g/L, which corresponds to EPS productivity of 0.2, 0.2, 0.2, and 0.1 g/d for pH 4, 5, 6, and 7, respectively. The maximal adenosine production in the culture broth was 42.3, 50.2, 78.4 and 120.5 mg/L, which corresponds to productivity of 7.8, 12.1, 15.1, and 20.7 mg/d for pH 4, 5, 6, and 7, respectively. The production of adenosine by Cordyceps sp. in submerged cultivation was scarcely studied. In addition, no adenosine was detected in the medium filtrate of the Cordyceps culture. To the best of our knowledge, the pH effects on the adenosine production in the liquid medium of *P. tenuipes* as described in this study are reported for the first in the literature, although the effects of dissolved oxygen (DO), carbon sources, and carbon/nitrogen on the adenosine production in the submerged cultivation of *P. tenuipes* have been investigated by many investigators. It should be noted that the direct comparison of metabolite productions of various Cordyceps strains from the various studies in the literature was difficult because the nutrient components and the culture conditions used were not exactly the same.

Effect of nitrogen sources on cell growth and the production of metabolites

The effects of nitrogen sources on the mycelial growth and the production of various metabolites by *P. tenuipes* in submerged cultivation are shown in Table 2. The organic nitrogen sources (peptone, yeast extract and soybean) gave rise higher mycelial growth. This is consistent with what was previously suggested that most basiomycetes prefer complex organic nitrogen sources for their growth in submerged cultures (Jennison et al., 1955). Several *P. tenuipes* species such as Cordyceps have been shown to have very poor mycelial growth in inorganic nitrogen sources (Dong and Yao, 2005). Therefore, only organic nitrogen sources were examined for their effects on the production of exopolysaccharide and adenosine by *P. tenuipes* in the medium. Amongst organic sources, yeast extract yielded the best EPS production (2.6 g/L). As shown in Table 2, the highest adenosine production (61.7 mg/L) in the medium was achieved when soybean was the nitrogen source; at the same time, the highest adenosine productivity (17.4 mg/d) was obtained when soybean was the nitrogen source. The systematic study of nitrogen sources on the production of cordycepin in the submerged culture was rarely seen, although there was a report indicating that peptone was a good nitrogen source for metabolite production by a strain of Cordyceps in submerged cultivation (Mao et al., 2005). We expect that soybean can replace peptone to enhance adenosine.

Based on the above results, soybean was selected as a suitable nitrogen source for further studies. Table 3 summarizes the effects of initial soybean concentrations on cell growth and adenosine production by *P. tenuipes*. The mycelial growth decreased with the increase of soybean concentration when the initial pH was kept at 6; the mycelia dry weight was 15.8, 11.1, and 6.6 mg/L when soybean concentration was 10, 15, and 30 g/L, corresponding to the C/N ratio (mass ratio) of 1/1, 1/1.5, 1/3, respectively. A previous study concerning the effect of the C/N ratio (mass ratio) on cell growth of a Cordyceps strain showed that a higher C/N ratio was preferred for the mycelial growth (Park et al., 2001), an observation that accorded with what was found in this work. The influence of C/N ratio on the production of adenosine by *P. tenuipes* strain has not been evaluated until now; the adenosine production was 61.7, 79.5, and 65.3 mg/L when soybean concentration was 10, 15, and 30 g/L, corresponding to the C/N ratio (mass ratio) of 1/1, 1/1.5, 1/3, respectively. This indicates that a lower C/N ratio is favorable for adenosine production; however, a high concentration of nitrogen source leading to a too low C/N ratio will inhibit their production

Table 4. Cell growth and metabolite production in shake and static culture of *P. tenuipes*

Cell growth and metabolites	Culture type	
	Shake	Static
Mycelia dry weight (g/L)	15.8±0.1	14.7±0.5
Adenosine production (mg/L)	61.7±0.5	140.0±1.7

Values are the Mean ± SD, and the data shown are average duplicate values. The culture time indicated in the parenthesis was when the maximum cell mass was reached (30 days).

Table 5. Result of three factor, Box-Behnken experimental design

Trial no.	X_1	X_2, %	X_3, day	Y_1, mg/L
1	6	3	16	53.43
2	7	3	20	120.96
3	7	3	20	125.12
4	6	4.5	20	50.76
5	8	3	16	72.14
6	7	3	20	131.92
7	8	4.5	20	60.24
8	8	3	24	74.04
9	6	3	24	57.36
10	7	3	20	124.6
11	7	1.5	24	78.36
12	7	4.5	16	80.21
13	7	3	20	135.4
14	7	4.5	24	94.08
15	7	1.5	16	75.48
16	6	1.5	20	49.32
17	8	1.5	20	58.69

X_1: Initial pH of the medium; X_2: Soybean concentration; X_3: day of shake culture; Y_1: Adenosine content

instead. Nevertheless, the data in Table 3 also reveal that the reduction of adenosine production by a significantly low C/N ratio can be offset by careful adjustment of medium pH. The above results indicate that adenosine biosynthesis is controlled by the balance between the culture conditions and nutrient concentrations.

Shake-flask and static culture of *P. tenuipes*

It is well-known that oxygen availability is critical to cell growth and metabolite formation in aerobic cell cultures. To investigate the effects of different fermentation methods on the mycelial growth and adenosine production by *P. tenuipes*, the cultivation was carried out in three different ways they are a shake-flask culture and a static culture, respectively. As shown in Table 4, the maximum mycelial production (15.8 g/L) was obtained at day 30 of cultivation in the shake-flask culture; in contrast, the maximum mycelial production (14.7 g/L) was obtained at day 30 of cultivation in the static culture. The adenosine production and productivity were 61.7 mg/L, and 140.0 mg/L for the static and the shake-flask culture, respectively. Obviously, the maximal adenosine production is higher in the static culture than in the shake-flask culture. In the previous study, it was seen that there were close relations between pellet size, cellular oxygen uptake, and Cordyceps production (Mao and Zhong, 2004). However, the relationships among cell morphology, oxygen uptake, and adenosine production still need to be determined.

Table 6. ANOVA for quadratic model on Box-Behnken experimental design

Source	F-value	p-value	
Model	51.71	<0.0001	Significant
A-pH	11.39	0.0118	
B-Soybean	2.13	0.1880	
C-Day of shake culture	1.97	0.2028	
AB	0.0001	0.9925	
AC	0.0319	0.8633	
BC	0.9354	0.3657	
A^2	267.90	<0.0001	
B^2	98.85	<0.0001	
C^2	42.45	0.0003	
Residual			
Lack of fit	0.8381	0.5394	Not significant
Pure error			
Cor Total			
$R^2 = 0.9852$			
Adjusted $R^2 = 0.9661$			
Predicted $R^2 = 0.8943$			

Optimization of adenosine production by RSM (Box–Behnken design)

It was reported that the complexities and uncertainties associated with large-scale fungi fermentation usually come from a lack of knowledge of the sophisticated interactions

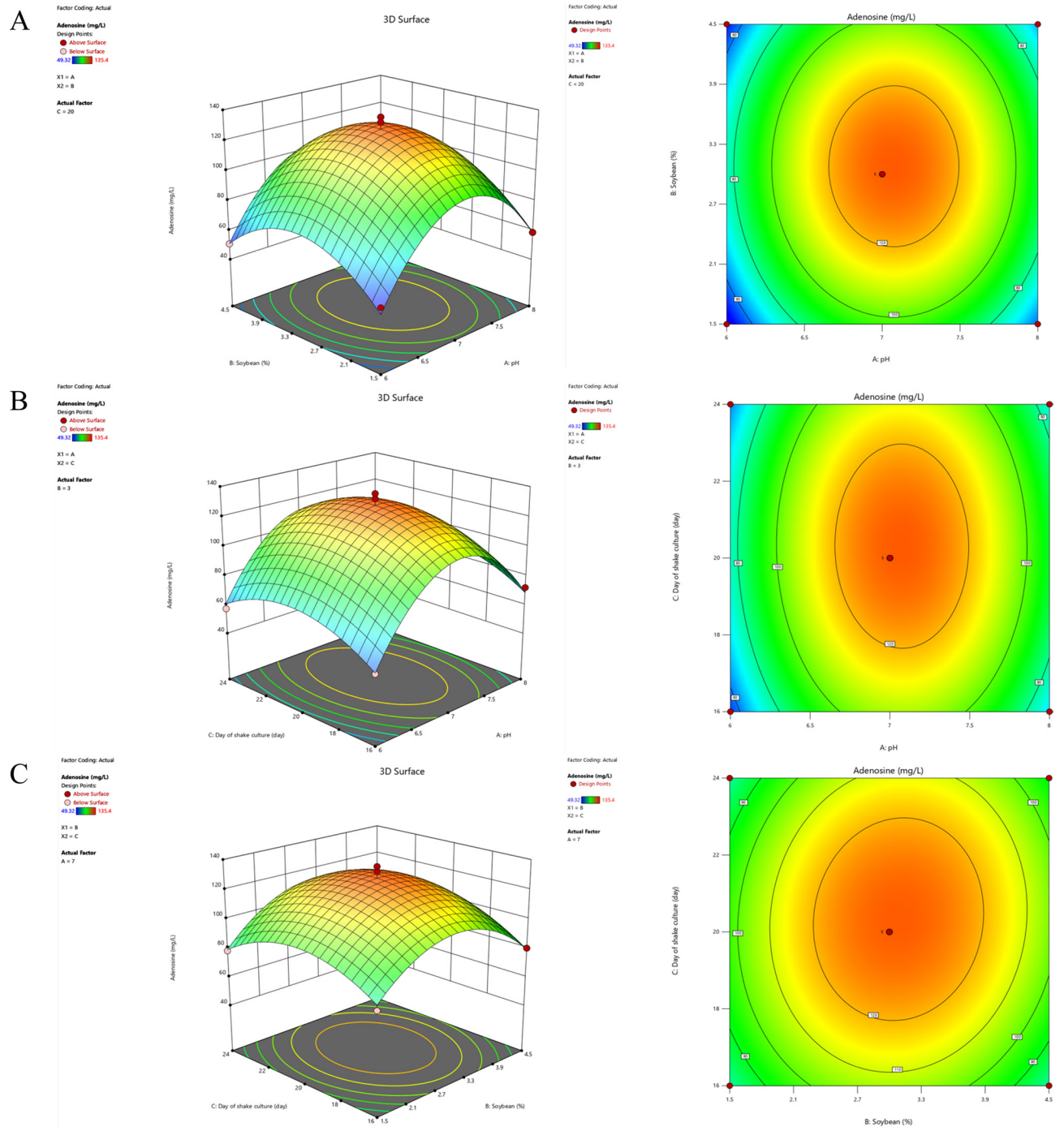

Fig. 1. 3D and contour map response surface plots for the adenosine content (Y_1). A: related with initial pH of the medium and soybean concentration; B: related with initial pH of the medium and day of shake culture; C: soybean concentration and day of shake culture.

among various factors. Our preliminary data shown above indicated that several major variables affect the performance of the culture in terms of adenosine production; they are the initial pH of the medium, the concentration of yeast extract, and the cultivation time of static culture. Once the variable having the greatest influence on the response was identified from the above results of the one-factor-at-a-time strategy, the Box–Behnken design (BBD) was used to optimize the levels of these variables. The four factors are initial pH (X_1), soybean concentration (X_2), and day of static culture (X_3). The levels of the variables for the BBD experiments were chosen in reconciliation with the data of our previous experiments on adenosine production by *P. tenuipes*. The BBD design and the corresponding experimental data are shown in Table 5.

By applying multiple regression analysis on the experimental data, the experimental results of the BBD design were fitted with a second-order polynomial equation (Eq. (1)). The results of the regression analysis are shown in Table 6, and the second-order polynomial equation obtained for adenosine production is shown in Eq. (2).

Second-order model equation:

$$Y\ (mg/L) = -2711.43750 + 643.72500X_1 + 66.07667X_2 + 45.31688X_3 + 0.018333\ X_1X_2 - 0.126875X_1X_3 + 0.457917X_2X_3 - 45.31875X_1^2 - 12.23500X_2^2 - 1.12742X_3^2 \quad \text{Eq. (2)}$$

This fit of the model was checked by the coefficient of determination R2, which was calculated to be 0.9852, indicating that 98.5% of the variability in the response could be explained by the model. The test statistics F values for the overall regression are significant at the upper 5% level, which further supports that the second-order model is very adequate in approximating the response surface of the experimental design. After performing the transformation of Eq. (2) to its canonical form, the optimum combination was found to be the following: pH 7; soybean concentration, 3 %; day of static culture, 20 days. The model predicted a maximum response of 128.05 mg/L adenosine yield. Verification of the calculated maximum was done with experiments that were performed in the culture media representing the optimum combination found, and the adenosine yield of 141.1 mg/L (average of three repeats) was obtained. Although the measured value did not justify the predicted value of the response model, it is, however, significantly higher than that, obtained by the one-factor-at-a-time method described above. Although adenosine can be synthesized chemically, such a route is cumbersome and requires complicated separation that leads to low yield and the use of a large volume of harmful organic solvents. Submerged cultivation of Cordyceps is seen as a promising alternative to chemical synthesis and soil cultivation for adenosine production. However, very low levels of adenosine were previously produced in mycelium and culture broth during submerged cultivation of Cordyceps sp.. Furthermore, adenosine has received less attention compared to cordycepin; therefore, this study will be used as important information for the production of adenosine from Cordyceps species.

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