Asian Journal of Mycology 8(1): 141–155 (2025) ISSN 2651-1339
www.asianjournalofmycology.org

Article
Doi 10.5943/ajom/8/1/10

Optimization for High-Yield Adenosine Production from *Cordyceps militaris* in Submerged Fermentation

Chuenprasert N [1,2], Yodsuwan N[1,2], Wei T[3,4] and Chamyuang S[1,2*]

[1]*School of Science, Mae Fah Luang University, Tha-Sut, Muang, Chiang Rai, 57100 Thailand*
[2]*Microbial Products and Innovation Research Group, Mae Fah Luang University, Chiang Rai, 57100 Thailand*
[3]*College of Food Science & Institute of Food Biotechnology, South China Agricultural University, Guangzhou, 510640, China*
[4]*Research Center for Micro-Ecological Agent Engineering and Technology of Guangdong Province, Guangzhou, 510640, China*

Chuenprasert N, Yodsuwan N, Wei T, Chamyuang S. 2025 – Optimization for High-Yield Adenosine Production from *Cordyceps militaris* in Submerged Fermentation. Asian Journal of Mycology 8(1), 141–155, Doi 10.5943/ajom/8/1/10

Abstract

Cordyceps militaris, a prized medicinal fungus in East Asian traditional medicine, is renowned for its bioactive compound, adenosine. This compound exhibits diverse pharmacological properties, including anti-aging, skin regeneration, wrinkle reduction, and hair growth promotion. To optimize adenosine production, we employed a Taguchi experimental design to evaluate the impact of key factors with the following conditions: fungal strains (*C. militaris* SH01, *C. militaris* ATCC 34165, and hybrid strain of *C. militaris* SH01 and *C. militaris* ATCC 34165), glucose concentration (20, 40, and 60 g/L), yeast extract concentration (5, 10, and 20 g/L), and initial pH (4.0, 5.5, and 7.0). The optimization experiments were carried out in 250-mL shaking flasks at 110 rpm at 25 °C. Among the tested strains, *C. militaris* SH01 demonstrated superior adenosine production under predicted optimal conditions from the Taguchi method (Qualitek-4 software): 40 g/L glucose, 20 g/L yeast extract, and an initial pH of 4.0. This resulted in a maximum adenosine yield of 8.662±0.269 mg/g and a productivity of 2.495±0.077 mg/g.d, surpassing the predicted values by 44%. However, when scaled up to a 5-L shaking flask, adenosine productivity decreased to 1.849±0.094 mg/g.d. Our findings provide valuable insights for enhancing adenosine production from *C. militaris*, paving the way for its potential application in various industries, including healthcare, food, and cosmetics.

Keywords – Adenosine – *Cordyceps militaris* – Process Optimization – Submerged Fermentation – Taguchi Method

Introduction

Cordyceps militaris, a medicinal fungus, has gained significant attention for its rich content of bioactive compounds, particularly adenosine and its derivative, cordycepin (3'–deoxyadenosine) (Jędrejko et al. 2021). Adenosine, a naturally occurring nucleoside in our bodies, is synthesized through a series of enzymatic reactions involving precursor molecules (Chang et al. 2024). In the cordycepin pathway, adenosine serves as a precursor of cordycepin production. During the early growth phase, adenosine is modulated by purine metabolism, then the gene cluster (*Cns1-4*) encodes the response enzyme to convert adenosine to cordycepin (Wei et al. 2025). This versatile compound holds immense potential for therapeutic applications across various industries, including medicine,

Submitted 21 January 2025, Accepted 26 May 2025, Published 12 June 2025
Corresponding Author: Sunita Chamyuang – e-mail – sunita@mfu.ac.th

pharmaceuticals, food, and cosmetics. The biosynthesis of these nucleosides is of significant interest due to their association with numerous health benefits, such as immunomodulation, anti-inflammatory, anticancer, antioxidant, anti-aging, skin regeneration, anti-wrinkle, and anti-hair loss properties (Chou et al. 2024, Kim et al. 2022, Marucci et al. 2022).

While *C. militaris* naturally produces adenosine in its fruiting bodies, whether cultivated on insect larvae or artificial media (Lim et al. 2012, Oh et al. 2019), its extended growth period of up to 60 days limits large-scale production. To meet the increasing demand for adenosine and cordycepin, researchers have turned to artificial media-based cultivation techniques, such as solid-state fermentation and liquid fermentation methods (Duan et al. 2023). Among these, submerged fermentation offers several advantages, including controlled environmental conditions, faster production cycles, higher yields, and scalability. Submerged fermentation can increase mycelial yield in a shorter time than solid-state fermentation, making it a suitable method for adenosine production, as adenosine is a growth-associated product. Adenosine is produced during the early stages of fungal growth but declines as cordycepin production increases. Submerged fermentation achieves the highest adenosine concentrations in *Cordyceps* species within 2–10 days (Ke & Lee 2019), whereas solid-state fermentation requires 40–50 days to reach comparable yields (Lim et al. 2012, Borde & Singh 2023). Various factors, including strain selection, medium composition (i.e. glucose and yeast extract concentrations), and the initial pH of the medium, play crucial roles in optimizing both biomass production and metabolite yields (Adnan et al. 2017, Ghatnur et al. 2015, Li et al. 2020, Patthanajuck & Bunnag 2021, Woraphokanunt et al. 2021).

While numerous studies have focused on optimizing submerged fermentation for cordycepin production, fewer have specifically targeted adenosine production on an industrial scale. This study aims to address this gap by optimizing the submerged fermentation process for *C. militaris* to maximize adenosine yield. A Taguchi experimental design was employed to identify and optimize critical factors influencing adenosine production. Although most studies focus on cordycepin production, studies on adenosine production under submerged fermentation have been largely neglected. Developing an optimized protocol for submerged fermentation could significantly enhance the industrial production of adenosine, thereby expanding its applications in the pharmaceutical and nutraceutical industries.

Materials & Methods

Microbial strains and culture conditions

Three *Cordyceps militaris* strains were used in this study: SH01 (Shanghai, China), ATCC 34165 (American Type Culture Collection), and a hybrid strain derived from SH01 and ATCC 34165. Strains were maintained on potato dextrose agar (PDA) plates at 20 °C and sub-cultured every 21 days. Stock cultures were kept in -80 °C.

Seed cultures were prepared by adding 10 discs (5 mm in diameter) of the active *C. militaris* mycelium into 500-mL Erlenmeyer flasks containing 200 mL of seed medium. The seed medium composition was as follows: 40 g/L glucose, 10 g/L yeast extract, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, and 0.5 g/L $MgSO_4 \cdot 7H_2O$. The initial pH was adjusted to 7.0. Flasks were incubated at 25° C and 150 rpm for five days (the method modified from Kang et al. 2014). All cultivation media and supplements were purchased from HiMedia Laboratories, LLC.

Optimization of submerged fermentation using Taguchi experimental design

To optimize adenosine production, a Taguchi experimental design was utilized. The analysis of experimental data and identification of optimal conditions were performed using Qualitek-4 software (Nutek Inc., Bloomfield Hills, MI, USA). An L_9 orthogonal array was employed, consisting of nine experimental runs with four factors tested at three levels each, as detailed in Table 1 and 2, respectively.

In addition to the variable parameters outlined in Table 1, the basal medium composition for all conditions consisted of 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, and 0.5 g/L $MgSO_4 \cdot 7H_2O$. The experiments were conducted in a 250-mL Erlenmeyer flask containing 50 mL of medium inoculated

with a 10% (v/v) seed culture of *C. militaris.* The cultures were incubated at 25 °C with shaking at 110 rpm for 30 days. Samples were collected every three days for adenosine analysis, and all experiments were performed in triplicate to ensure statistical validity.

Table 1 The factors and their levels for optimization of adenosine production

Factor	Level		
	1	**2**	**3**
A: Fungal strain (*C. militaris*)	SH01	ATCC 34165	Hybrid
B: Glucose concentration (g/L)	20	40	60
C: Yeast extract concentration (g/L)	5	10	20
D: Initial pH	4.0	5.5	7.0

Following the cultivations conducted according to the Taguchi-based experimental design, the adenosine content from each experimental run was analyzed and input into Qualitek-4 software. This analysis facilitated the identification of the optimal conditions for adenosine production in *C. militaris*. Subsequently, experiments based on the identified optimal conditions were performed in triplicate. This step is essential to validate the adenosine production levels before proceeding with the scale-up experiments.

Scale-up of optimal conditions in a 5-L shaking flask

To assess the scalability of the optimal conditions identified from the previous section, a 5-L shaking flask was utilized, containing 3.5 L of the optimized medium. A 10% (v/v) inoculum of the seed culture was added to this medium, resulting in a final volume of 3.5 L. The *C. militaris* culture was incubated at 25 °C with rotary shaking at 150 rpm, without pH control, for a period of five days. Samples were harvested from each flask to facilitate analysis. The parameters measured included adenosine content, dry cell weight, residual glucose concentration, and pH levels, with all measurements conducted in triplicate.

Determination of mycelial dry weight, pH, and residual glucose

For analysis, 50 mL samples comprising both mycelium and supernatant were collected from each flask every three days. The samples were centrifuged at 8000 rpm for 15 minutes (MPW-352R Refrigerated Laboratory Centrifuge, Poland) to separate the mycelium from the supernatant. In addition to this, mycelium harvested from the 5-L shaking flask was also processed. The mycelium was filtered using a 45 µm nylon membrane and thoroughly rinsed with distilled water. It was then freeze-dried prior to determining dry cell weight and extracting adenosine yield using the methods described below.

The supernatant was analyzed for pH using a calibrated pH meter, and residual glucose concentration was determined utilizing the DNS method as modified by Chuenprasert et al. (2021) (originally described by Miller (1959)). Specifically, residual glucose was quantified by adding 0.5 mL of DNS solution to 0.5 mL of supernatant. The mixture was incubated at 95 °C for 5 minutes and then allowed to cool for an additional 5 minutes. Absorbance was measured at 540 nm using a microplate reader (Biotek Synergy HT Multi-Mode Microplate Reader, USA). A standard curve was constructed using glucose concentrations ranging from 0.2 to 1.0 mg/mL, and residual glucose concentrations were calculated using the linear equation derived from this calibration curve.

Adenosine and Cordycepin extraction from *C. militaris*

Adenosine and cordycepin were extracted from *C. militaris* and quantified using high-performance liquid chromatography (HPLC), following the protocol established by ChokeUmnuay & Owatworakit (2021). For the extraction process, 50 mg of freeze-dried mycelium was macerated with 1 mL of distilled water (dH_2O) and incubated at 80 °C for 3 hours. The resulting mixture was then centrifuged at 8000 rpm for 15 minutes at 4 °C to separate the supernatant, which was

subsequently filtered through a 0.2 µm nylon syringe filter. A volume of 1 µL from the filtered solution was utilized for analysis via high-performance liquid chromatography (HPLC).

HPLC analysis for Adenosine and Cordycepin content

An aliquot of the crude extract (1 µL) was analyzed using high-performance liquid chromatography (HPLC) on a Waters ACQUITY Arc System (Waters Corporation, Milford, USA), equipped with a photodiode array (PDA) detector and operated via Empower 3 Software. The analysis utilized a Kinetex C_{18} reverse-phase column (4.6 × 250 mm, 5 µm; Phenomenex, Inc., Torrance, California, USA). The mobile phase consisted of solvent A (methanol) and solvent B (0.2% formic acid aqueous solution) in a volumetric ratio of 95:5 (V/V), employing an isocratic elution at a flow rate of 0.2 mL/min. The column was maintained at 30 °C, with adenosine detected at an absorption wavelength of 260 nm. For calibration, adenosine and cordycepin standards (Sigma–Aldrich; USA) were prepared in distilled water across eight concentrations ranging from 100 to 0.78 µg/mL. The calibration curve was generated by plotting peak areas against the corresponding concentrations of each standard solution, demonstrating linearity with a correlation coefficient $r^2 > 0.999$.

Kinetic parameter estimation

The yield of adenosine ($Y_{Ade/X}$) was calculated as follows:

$$Y_{\mathrm{Ade/X}} = \frac{C_{\mathrm{Ade}}}{C_{\mathrm{X}}}$$

where $Y_{Ade/X}$ is the yield of adenosine from freeze–dried mycelium (mg/g), C_{Ade} is the concentration of adenosine (mg/mL), and C_X is the cell concentration (g/mL).

The productivity of adenosine ($Q_{P,Ade}$) was calculated as follows:

$$Q_{\mathrm{P,Ade}} = \frac{Y_{\mathrm{Ade/X(a)}} - Y_{\mathrm{Ade/X(b)}}}{t_{(\mathrm{a})} - t_{(\mathrm{b})}}$$

where $Q_{P,Ade}$ is the productivity of adenosine (mg/g. d), $Y_{Ade/X}$ is the yield of adenosine from freeze-dried mycelium (mg/g), t is time (d), and a, b represent the date of harvesting.

Statistical analysis

Experimental and statistical analysis was calculated using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) of data and graphics were generated by Qualitek-4 software (Nutek Inc., Bloomfield Hills, MI, USA) and Microsoft Excel 365, respectively. Data were presented as mean ± standard deviation.

Results and Discussion

The production of Adenosine under submerged fermentation in 250-mL shaking flasks

The effect of each factor including fungal strain (A), glucose concentration (g/L) (B), yeast extract concentration (g/L) (C), and initial pH (D) at the assigned level was used for enhancing the productivity of adenosine ($Q_{P,Ade}$) under submerged fermentation of *C. militaris*. The nine different experiments were carried out with the factors at the levels specified by the L_9 (3^4) orthogonal array, as shown in Table 2. For the production of adenosine, the maximum yield of adenosine ($Y_{Ade/X,max}$) was observed in treatment 5, reaching 4.080±0.415 mg/g on day 3 (Fig. 1). Furthermore, to indicate the efficiency of the process, productivity was carried out. The maximum productivity of adenosine ($Q_{P,Ade,max}$; 1.360±0.204 mg/g.d) was also obtained in treatment 5, in which condition the *C. militaris* strain ATCC 34165 was grown under glucose 40 g/L, yeast extract 20 g/L, and an initial pH of 4.0.

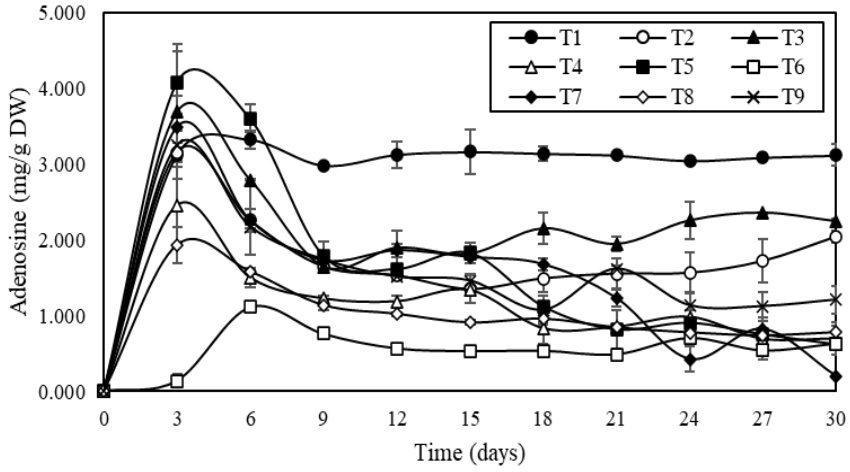

Fig. 1 – Yield of adenosine ($Y_{Ade/X}$) by various conditions cultivated in 250-mL shaking flasks

Table 2 The factor levels and productivity of adenosine ($Q_{P,Ade}$) for the different treatments

Treatment	Factor *				Productivity of adenosine ($Q_{P,Ade}$) (mg/g.d) **
	A	B	C	D	
1	SH01	20	5	4.0	1.043±0.004 [bc]
2	SH01	40	10	5.5	1.054±0.042 [bc]
3	SH01	60	20	7.0	1.231±0.216 [ab]
4	ATCC 34165	20	10	7.0	0.820±0.120 [cd]
5	ATCC 34165	40	20	4.0	1.360±0.204 [a]
6	ATCC 34165	60	5	5.5	0.046±0.025 [e]
7	Hybrid	20	20	5.5	1.163±0.137 [ab]
8	Hybrid	40	5	7.0	0.644±0.079 [d]
9	Hybrid	60	10	4.0	1.082±0.063 [abc]

Note * A, Fungal strain; B, glucose concentration (g/L); C, yeast extract concentration (g/L); D, initial pH.

** The mean difference is significant at the 0.01 level.

Influence of factors on Adenosine production in 250-mL shaking flasks: Interaction effect and analysis of variance

The productivity of adenosine ($Q_{P,\ Ade}$) was calculated to analyze the main effects of individual factors, their interaction effects, and to perform the variance analysis (Table 3). To determine the most significant factor, the main effect of each factor was expressed as a percentage, calculated by dividing its individual effect by the sum of the main effects of all factors. The results indicated that the concentration of yeast extract (g/L) (factor C) was the most significant factor affecting adenosine production, accounting for 40.071% of the total variability. This finding shows the importance of nutrient availability in microbial fermentation, as yeast extract serves as a rich source of amino acids, vitamins, and growth factors that can enhance microbial metabolism and, consequently, metabolite production (Yang et al. 2014, Zarei et al. 2016). The report suggested that yeast extract was the most effective for promoting *C. cicadae* mycelial growth and adenosine yield (3.416 mg/g), compared to ammonium citrate, diammonium oxalate monohydrate, and peptone (Zhu et al. 2024). An initial pH (factor D) and fungal strain (factor A) were also significant contributors, with main effects of 24.197% and 21.879%, respectively. The initial pH is particularly crucial, as it can influence enzyme activity and metabolic pathways in the fungal strains used for fermentation (Adnan et al. 2017).

However, the effect of initial pH on adenosine production by *Cordyceps* sp. has been scarcely studied. A study on *Paecilomyces tenuipes* found that relatively low initial pH levels were optimal

for adenosine production (120.5 mg/L), with the best results observed at a pH of 7.0 (Ha et al. 2024). The growth of mycelium, which is crucial for metabolite production, is also affected by pH. Acidic conditions generally promote better mycelial growth and subsequent metabolite production (Rózsa & Apahidean 2020). The moderate effect of the fungal strain suggests that while different strains may have varying efficiencies in metabolite production, their performance is heavily influenced by regions as well as environmental conditions such as nutrient concentration and pH (Kontogiannatos et al. 2021, Liu et al. 2017). Notably, genetic engineering approaches have been successfully employed to enhance adenosine production in other organisms like *Bacillus subtilis*, where adenosine production was increased from 7.40 to 14.39 g/L through upregulation of the *pur*A gene (Li et al. 2019). Similar strategies could be applied to *C. militaris* to improve adenosine yields. In contrast, glucose concentration (factor B) exhibited a lower main effect (13.853%), indicating that while it is necessary for energy supply (Patthanajuck & Bunnag 2021), its impact on adenosine production is relatively limited. Glucose plays a supportive role by being converted to ribose-5-phosphate (R-5-P), a precursor in the purine pathway that leads to the synthesis of nucleotides like IMP and AMP, and ultimately resulting in adenosine (Chang et al. 2024). Although glucose provides essential resources for energy and carbon supply, the specific transcriptional regulation and metabolic flux towards adenosine synthesis might not be as strongly influenced by glucose as it is for cordycepin. Besides, its effects might be overshadowed by other factors like nitrogen source and other supplements (Ke & Lee 2019). This may suggest that once an adequate level of glucose is provided, further increases do not significantly enhance adenosine production.

Table 3 The main effect of each factor on the productivity of adenosine

Level	Factor [a]			
	A	**B**	**C**	**D**
Productivity of adenosine ($Q_{P,Ade}$) (mg/g.d)				
1	1.109	1.008	0.577	1.161
2	0.741	1.019	0.985	0.754
3	0.963	0.786	1.251	0.898
Minimum value	0.741	0.789	0.577	0.754
Maximum value	1.109	1.019	1.251	1.161
Main effect [b]	0.368	0.233	0.674	0.407
% Main effect [c]	21.879	13.853	40.071	24.197

Note [a] A, Fungal strain; B, glucose concentration (g/L); C, yeast extract concentration (g/L); D, initial pH.

[b] Main effect = maximal value – minimal value.

[c] % Main effect = (100 × main effect)/total main effect. Total main effect is 1.682.

The severity index (SI) quantifies the interaction effects of two individual factors on adenosine production (Table 4). The highest SI value (50.39%) was observed between the fungal strain and initial pH, indicating a significant interaction that moderately influenced adenosine production. In the preliminary study, the initial pH showed a lesser effect on adenosine production but showed a greater impact on cordycepin production (data not shown). However, the interaction effect between fungal strain and initial pH showed the most significant effect due to the profound impact of pH on fungal growth dynamics, enzyme functionality, and strain-specific adaptations (Gazengel et al. 2020). The precise control of initial pH is necessary to boost desirable metabolite production in the appropriate strain. Additionally, yeast extract is a complex source of nutrients that is widely used in microbial fermentation processes to promote growth and enhance the desired metabolite production. By optimizing the concentration of yeast extract, it was identified as the most impactful factor (Table 3), particularly when interacting with initial pH (36.82%), fungal strain (29.05%), and glucose concentration (24.08%). Although glucose concentration was noted as a less impactful factor (Table 3), it still demonstrated interactions with other factors; it showed a SI value of 20.16% with the fungal strain and 16.24% with the initial pH (Table 4). However, adenosine production relies more on the

interactions between various factors than on their individual effects. Optimizing these parameters enhances fermentation efficiency and increases adenosine yields, highlighting the importance of a multifactorial approach in fermentation processes.

Table 4 Estimated interaction severity index (SI) of the factors that affect adenosine production by *C. militaris* under optimal conditions of submerged fermentation

No.	Interacting Factor Pairs	Columns [a]	SI (%) [b]	Col [c]	Opt.[d]
1	Fungal strain x Initial pH	1 x 4	50.39	5	[2,1]
2	Yeast extract concentration x Initial pH	3 x 4	36.82	7	[3,1]
3	Fungal strain x Yeast extract concentration	1 x 3	29.05	2	[2,3]
4	Glucose concentration x Yeast extract concentration	2 x 3	24.08	1	[2,3]
5	Fungal strain x Glucose concentration	1 x 2	20.16	3	[2,2]
6	Glucose concentration x Initial pH	2 x 4	16.24	6	[2,1]

Note [a] Represent the column locations to which the interacting factors are assigned.

[b] Interaction severity index (100% for 90^o angle between the lines, 0% for parallel lines)

[c] Shows column that should be reserved if this interaction effect were to be studied (2-L factors only).

[d] Indicates the factor levels desirable for the optimum condition (based strictly on the first 2 levels)

The analysis of variance (ANOVA) (Table S1, supplementary data) further confirmed these findings, revealing that yeast extract concentration had a statistically significant effect on adenosine productivity ($p < 0.01$), contributing to 50.907% response variability. In contrast, the contributions from initial pH (18.444%), fungal strain (14.638%), and glucose concentration (7.026%) were comparatively lower but still relevant. These results indicate that while optimizing yeast extract concentration is crucial for maximizing adenosine yields, attention must also be given to adjusting initial pH and selecting appropriate fungal strains to exploit their synergistic effects. Future studies should focus on fine-tuning these parameters and investigating new interactions to improve production processes in fermentation. From these findings, *C. militaris* SH01 cultured under 40 g/L glucose, 20 g/L yeast extract, and an initial pH 4.0 condition (Fig. 2) was suggested as the predicted optimal condition for mycelium formation and adenosine production.

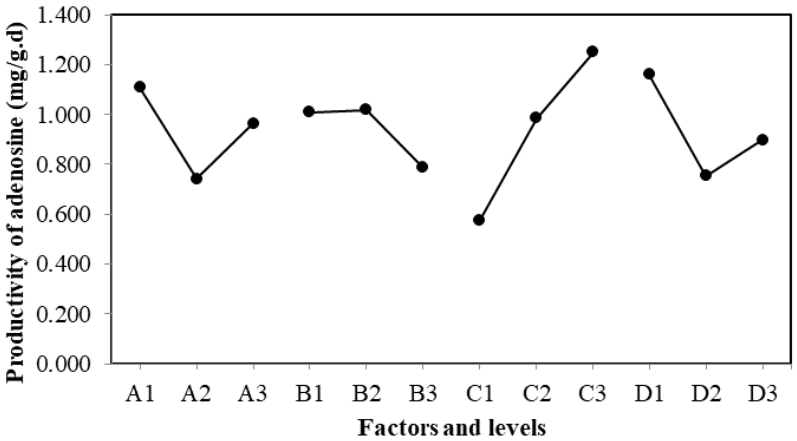

Fig. 2 – Productivity of adenosine for the various factors and levels: A, Fungal strain; B, glucose concentration (g/L); C, yeast extract concentration (g/L); D, initial pH

Optimization of Adenosine production and confirmation under 250-ml shaking flask

The study identified optimal conditions for maximizing adenosine production using *C. militaris*: A1 (A=SH01), B2 (B=40 g/L), C3 (C=20 g/L) and D1 (D=pH 4.0) (Fig. 2). Over approximately three days, glucose was gradually consumed, leading to the maximum cell concentration ($C_{X,max}$) of 26.295±0.626 g/L at day 5 (Fig. 3A). The maximum adenosine yield ($Y_{Ade/X,max}$) was obtained on the second day at 8.662±0.269 mg/g but declined thereafter, while cordycepin production began to rise exponentially from the fifth day, reaching a maximum of 6.054±0.603 mg/g on the seventh day (Fig. 3B), indicating a relationship where a decrease in adenosine correlated with an increase in cordycepin levels. According to the biosynthetic pathway of cordycepin, adenosine was used as a precursor of cordycepin and pentostatin via the alternative functions of the N-terminal NK and C-terminal HisG domains of *Cns3*. In addition, adenosine deaminase (ADA), which is the crucial enzyme to converts adenosine to inosine, reduces adenosine levels. The ADA can also degrade cordycepin into 3'-deoxyinosine, which may counteract some of the increases in cordycepin levels if ADA activity is not effectively inhibited by pentostatin (Turk et al. 2023, Xia et al. 2017, Zeng et al. 2024). For the specified factor values, the predicted adenosine productivity under these optimal conditions was 1.727 mg/g.d, but validation experiments showed a higher measured productivity ($Q_{P,Ade}$) of 2.495±0.077 mg/g.d, indicating a 1.44-fold increase over predictions (Table 5).

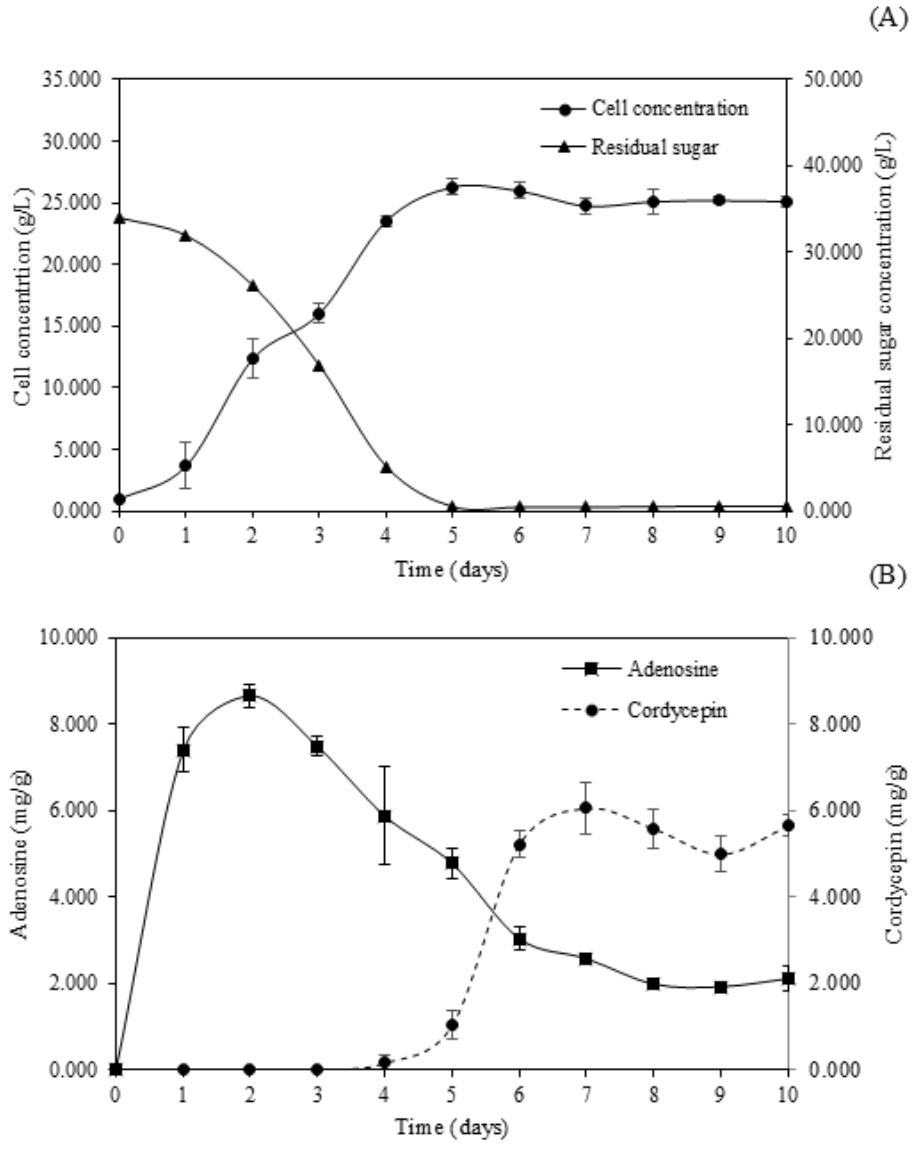

Fig. 3 – (A) The changes of cell concentration and residual glucose, and (B) yield of adenosine and cordycepin under optimal conditions cultivated in a 250-mL shaking flask.

Table 5 Productivity of adenosine under optimal conditions

Working volume (mL)	Optimal conditions [a]				Productivity of adenosine ($Q_{p,Ade}$) (mg/g.d)	
	A	B	C	D	Expected result [b]	Measured value [c]
50	SH01	40	20	4.0	1.727	2.495±0.077
3500	SH01	40	20	4.0	1.727	1.849±0.094

Note [a] A, Fungal strain; B, Glucose concentration (g/L); C, Yeast extract concentration (g/L); D, Initial pH.

[b] Expected result was calculated as $Y_{expected} = \overline{T} + (\overline{A}_{opt} - \overline{T}) + (\overline{B}_{opt} - \overline{T}) + (\overline{C}_{opt} - \overline{T}) + (\overline{D}_{opt} - \overline{T})$. The grand average of performance, $\overline{T}$, was 0.938.

[c] Calculated based on the yield of adenosine on day 3 of fermentation.

In this present work, the optimal yeast extract concentration was 20 g/L, while the lower concentrations (5 and 10 g/L) proved less desirable. It is different from (Ke & Lee 2019), who reported that an addition of 0.2% yeast extract in the production medium (PDB) enhances adenosine production in *C. cicadae* NTTU 868. The different fungal strains may have different nutritional requirements and preferences for yeast extract. Optimizing the concentration of yeast extract for a specific strain can help maximize its growth and metabolite production. Previous studies suggested that the highest adenosine content was obtained in a medium supplemented with 10 g/L of yeast extract for the BH strain, whereas the DA strain achieved the highest yield with 20 g/L peptone (Patthanajuck & Bunnag 2021). Likewise, the optimal initial pH for *Cordyceps* growth remains unclear and needs further investigation. In this study, an initial pH of 4.0 was proved to be optimal for adenosine production, as displayed in Fig. 2, whereas the initial pH levels of 5.5 and 7.0 were not suitable. The previous work reported that an acidic pH promotes mycelium growth and metabolite production in ascomycetes and basidiomycetes, while an optimal pH range of 6.0–7.0 is suggested for general growth (Adnan et al. 2017, Yang et al. 2014). In terms of glucose concentration, glucose concentrations at 20–60 g/L had a relatively limited effect on adenosine production during submerged fermentation of *C. militaris*. However, an initial concentration of 40 g/L was determined to be optimal in this study. According to Liu et al. (2019), a glucose concentration of 40 g/L can promote mycelium growth; however, its effect on adenosine production was not detailed. Furthermore, the balance between carbon and nitrogen sources is crucial for efficient fermentation and metabolite accumulation. Solakov et al. (2022) showed an adenosine yield of 8.46 mg/g for three days of cultivation, which is consistent with our results.

Scale-up of the Adenosine Production in a 5L Shaking Flask

The scalability of *C. militaris* under submerged fermentation was confirmed in a 5L shaking flask with a working volume of 3.5 L, using optimal conditions identified through a Taguchi experimental design (*C. militaris* SH01, 40 g/L glucose, 20 g/L yeast extract and pH 4.0). During cultivation, mycelium growth increased steadily while residual glucose decreased slightly, reaching a cell concentration (C_X) of 15.685 g/L by the end of fermentation (Fig. 4A). The maximum adenosine yield ($Y_{Ade/X,max}$) rose with mycelium development, peaking at 7.967±0.674 mg/g on the first day before declining slightly after day two (Fig. 4B). However, the cordycepin was not detected during the first five days of fermentation (Fig. 4B). During the early growth stages of fungi, adenosine is synthesized as a primary metabolite via purine metabolism pathways. It serves as a precursor to ATP, the primary energy molecule in cells, which supports vital cellular activities, including mycelium growth and nutrient uptake. Moreover, adenosine plays a role as a metabolic precursor and regulatory component in cordycepin biosynthesis, which may explain its decline after day one (Solakov et al. 2022, Wei et al. 2025). The absence of cordycepin during the first five days of fermentation suggests that cordycepin, which is a secondary metabolite, is synthesized primarily after the exponential growth phase, when the fungus enters the stationary phase, which may be typically due to stress responses or nutrient limitations (Bertrand 2019, Hamill et al. 2020). In validation experiments under optimal conditions, adenosine productivity ($Q_{P,Ade}$) was measured at 1.849±0.094 mg/g.d in the 5-L flask, which was lower than that observed in a 250-mL flask; however, it was found to be higher than the expected result by about 1.07-fold (Table 5). The findings suggested that both cell concentration (C_X) and adenosine productivity ($Q_{P,Ade}$) were inferior in the larger volume compared to the smaller one. As the fermentation volume increases, maintaining optimal conditions for mycelium growth and metabolite productivity becomes more complex. Key factors including nutrient distribution, oxygen availability, and environmental stability, i.e., temperature and pH control, during the process should be optimized at larger scales. In larger-volume fermentations, nutrients may not be evenly distributed, leading to areas with insufficient nutrients for optimal cell growth and productivity. Moreover, it can have lower oxygen concentrations at the bottom or in the center of the vessel due to diffusion limitations, further affecting cellular metabolism and productivity (Kang et al. 2014, Wen et al. 2017, Zhang & Zhong 2013). Additionally, adenosine production peaks early during the growth phase and then declines as biomass increases, indicating that oxygen

requirements for both growth and metabolite synthesis vary throughout the fermentation cycle (Ghatnur et al. 2015).

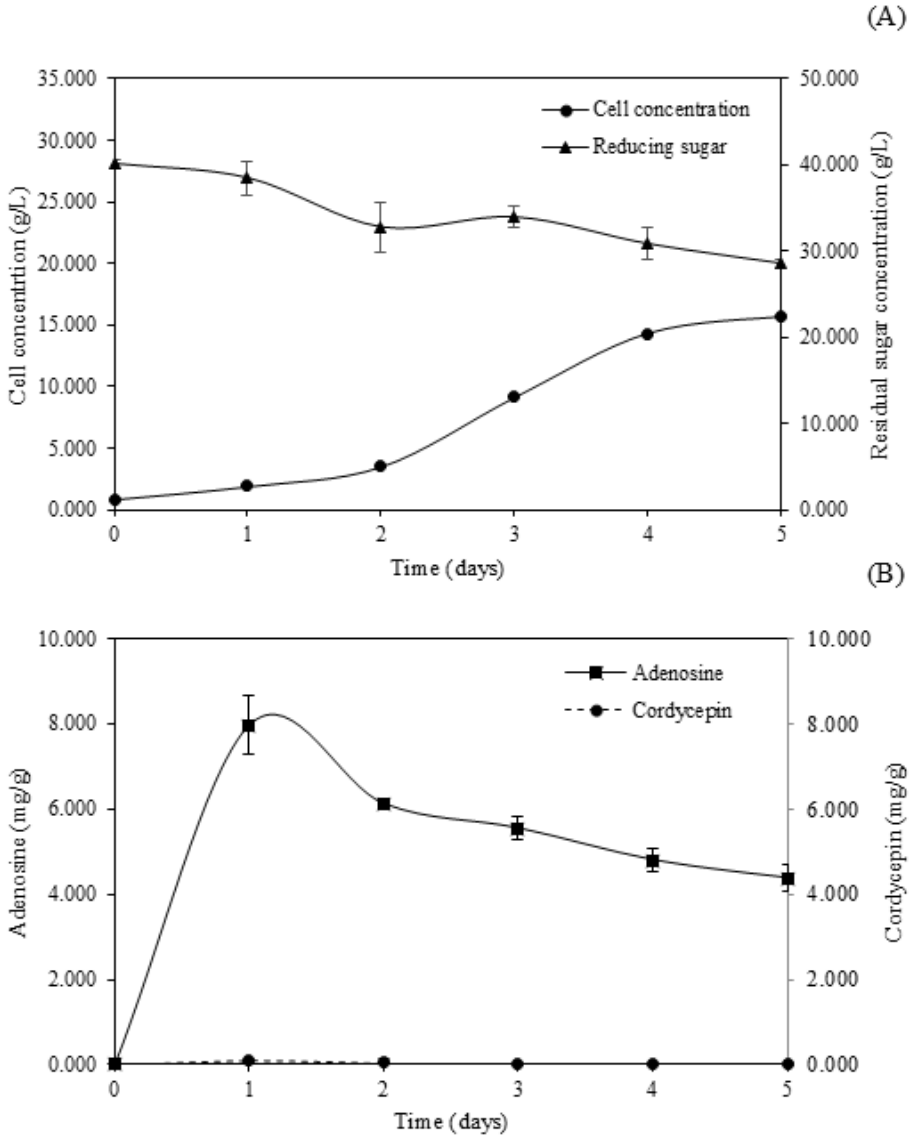

Fig. 4 – (A) The changes of cell concentration and residual glucose, and (B) yield of adenosine and cordycepin under optimal conditions cultivated in a 5-L shaking flask.

Conclusion

This investigation identified optimal conditions for adenosine production by *Cordyceps militaris* SH01 in submerged fermentation, revealing yeast extract concentration as the most influential factor among those tested (fungal strain, glucose concentration, yeast extract concentration, initial pH). Maximal adenosine productivity ($Q_{P,Ade}$= was 2.495±0.077 mg/g.d), achieved in 250-mL shake flasks using 40 g/L glucose, 20 g/L yeast extract, and an initial pH of 4.0 (50 mL volume), represented a significant 1.44-fold increase over predicted yields. Despite this success at the bench scale, transitioning the process to 5-L shaking flasks resulted in diminished performance, evidenced by lower adenosine productivity (1.849 ± 0.094 mg/g·d) and reduced final biomass concentration (15.685 g/L compared to 26.295 ± 0.626 g/L in 250-mL flasks). This difference underscores common challenges encountered during bioprocess scale-up, indicating issues with nutrient distribution, insufficient oxygen supply, or suboptimal environmental control (e.g., temperature gradients, pH fluctuations) within the larger volume. Future research should prioritize addressing these scale-dependent factors to enhance both mycelial growth and adenosine synthesis

in larger bioreactors. Overcoming these limitations is crucial for realizing the potential of *C. militaris* as an efficient source of adenosine for industrial applications in healthcare, food, and cosmetics.

Acknowledgements

The authors wish to express their gratitude to Mae Fah Luang University (MFU). We acknowledge the Global Relations Division and the Office of Postgraduate Studies for their financial support towards tuition fees. Research funding was provided by the Microbial Products and Innovation (MP&I) Research Group, School of Science, MFU. We also thank the Scientific and Technological Instruments Centre (STIC) for providing essential laboratory space and equipment for this study

References

Adnan M, Ashraf SA, Khan S, Alshammari E, Awadelkareem AM. 2017 – Effect of pH, temperature and incubation time on cordycepin production from *Cordyceps militaris* using solid-state fermentation on various substrates. CyTA-Journal of Food 15, 617–621.

Bertrand RL. 2019 – Lag phase is a dynamic, organized, adaptive, and evolvable period that prepares bacteria for cell division. Journal of Bacteriology 201(7), 10–1128.

Borde M, Singh SK. 2023 – Enhanced production of cordycepin under solid-state fermentation of *Cordyceps militaris* by using combinations of grains/substrates. Brazilian Journal of Microbiology 54(4), 2765–2772.

Chang Y, Liu X, Jiao Y, Zheng X. 2024 – Improved cordycepin production by *Cordyceps militaris* using corn steep liquor hydrolysate as an alternative protein nitrogen source. Foods 13, 813.

ChokeUmnuay N, Owatworakit A. 2021 – Effect of successive subculture of *Cordyceps militaris* on growth, metabolites production and stability of *Rhf1* gene. Agriculture and Natural Resources 55, 537–546.

Chou YC, Sung TH, Hou SJ, Khumsupan D et al. 2024 – Current progress regarding *Cordyceps militaris*, its metabolite function, and its production. Applied Sciences 14, 4610.

Chuenprasert N, Kookkhunthod T, Deeklom R, Yodsuwan N. 2021 – Influence of temperature on amylase enzyme profile during germination of two upland rice varieties. Asia-Pacific Journal of Science and Technology 26, 1–10.

Duan X, Yang H, Wang C, Liu H, Lu X et al. 2023 – Microbial synthesis of cordycepin, current systems and future perspectives. Trends in Food Science & Technology 132, 162–170.

Gazengel K, Lebreton L, Lapalu N, Amselem J et al 2020 – pH effect on strain-specific transcriptomes of the take-all fungus. Plos One 15(7), e0236429.

Ghatnur SM, Parvatam G, Balaraman M. 2015 – Culture conditions for production of biomass, adenosine, and cordycepin from *Cordyceps sinensis* CS1197: Optimization by desirability function method. Pharmacognosy Magazine 11, S448.

Ha SY, Kim HC, Lim WS, Yang JK. 2024 – Effects of culture conditions on the adenosine production in submerged culture of *Paecilomyces tenuipes*. Journal of Mushroom 22, 73–80.

Hamill PG, Stevenson A, McMullan PE, Williams JP et al. 2020 – Microbial lag phase can be indicative of, or independent from, cellular stress. Scientific Reports 10(1), 5948.

Jędrejko KJ, Lazur J, Muszyńska B. 2021 – *Cordyceps militaris*: an overview of its chemical constituents in relation to biological activity. Foods 10, 2634.

Kang C, Wen TC, Kang JC, Meng ZB et al. 2014 – Optimization of large-scale culture conditions for the production of cordycepin with *Cordyceps militaris* by liquid static culture. The Scientific World Journal 2014.

Ke BJ, Lee CL. 2019 – Using submerged fermentation to fast increase N6-(2-hydroxyethyl)-adenosine, adenosine and polysaccharide productions of *Cordyceps cicadae* NTTU 868. Amb Express 9, 198.

Kim J, Shin JY, Choi YH, Kang NG, Lee S. 2022 – Anti-hair loss effect of adenosine is exerted by cAMP mediated *Wnt/β*-catenin pathway stimulation via modulation of *Gsk3β* activity in cultured human dermal papilla cells. Molecules 27, 2184.

Kontogiannatos D, Koutrotsios G, Xekalaki S, Zervakis GI. 2021 – Biomass and cordycepin production by the medicinal mushroom *Cordyceps militaris*—a review of various aspects and recent trends towards the exploitation of a valuable fungus. Journal of Fungi 7, 986.

Li B, Yan ZY, Liu XN, Zhou J et al. 2019 – Increased fermentative adenosine production by gene-targeted *Bacillus subtilis* mutation. Journal of Biotechnology 298, 1–4.

Li JF, Hoang VA, Ahn JC, Yang DU et al. 2020 – Isolation of new strain of *Cordyceps militaris* HB8 and optimal condition for production of adenosine and cordycepin in fruit body. Korean Journal of Plant Resources 33, 696–706.

Lim L, Lee C, Chang E. 2012 – Optimization of solid state culture conditions for the production of adenosine, cordycepin, and D-mannitol in fruiting bodies of medicinal caterpillar fungus *Cordyceps militaris* (L.: Fr.) Link (*Ascomycetes*). International Journal of Medicinal Mushrooms 14, 181–187.

Liu K, Wang F, Wang W, Dong C. 2017 – *Beauveria bassiana*: a new N-6-(2-hydroxyethyl)-adenosine producing fungus. Mycology 8, 259–266.

Liu XC, Li H, Kang T, Zhu ZY, et al. 2019 – The effect of fermentation conditions on the structure and anti-tumor activity of polysaccharides from *Cordyceps gunnii*. RSC Advances 9, 18205–18216.

Marucci G, Buccioni M, Varlaro V, Volpini R, Amenta F. 2022 – The possible role of the nucleoside adenosine in countering skin aging: A review. BioFactors 48, 1027–1035.

Miller GL. 1959 – Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry 31, 426–428.

Oh J, Yoon DH, Shrestha B, Choi HK, Sung GH. 2019 – Metabolomic profiling reveals enrichment of cordycepin in senescence process of *Cordyceps militaris* fruit bodies. Journal of Microbiology 57, 54–63.

Patthanajuck V, Bunnag S. 2021 – Effects of carbon and nitrogen sources on fruiting body formation and cordycepin production of *Cordyceps militaris* (L.) Link. Khon Kaen Agriculture Journal 49, 274–283.

Rózsa M, Apahidean M. 2020 – Influence of temperature and pH level on mycelial growth in liquid cultures of *Cordyceps militaris* mushroom mycelium. Current Trends in Natural Sciences 9, 42–46.

Solakov N, Kostova M, Loginovska K, Markov Z et al. 2022 – Investigation of adenosine precursors and biologically active peptides in cultured fresh mycelium of wild medicinal mushrooms. Applied Sciences 12, 10618.

Turk A, Lee S, Yeon SW, Ryu SH et al. 2023 – Adenosine deaminase inhibitory activity of medicinal plants: boost the production of cordycepin in *Cordyceps militaris*. Antioxidants 12, 1260.

Wei T, Zeng J, Owatworakit A, Chamyuang S. 2025 – *Cordyceps militaris*: A microbial cell factory for the production of cordycepin. In Cordyceps and Allied Species. Singapore: Springer Nature Singapore 337–359.

Wen TC, Long FY, Kang C, Wang F, Zeng W. 2017 – Effects of additives and bioreactors on cordycepin production from *Cordyceps militaris* in liquid static culture. Mycosphere 8, 886–898.

Woraphokanunt Y, Jatupornpipat M, Rittiboon A. 2021 – Optimum carbon and nitrogen sources for enhancing bioactive compound production of *Isaria tenuipes*. Burapha Science Journal 1683–1691.

Xia Y, Luo F, Shang Y, Chen P et al. 2017 – Fungal cordycepin biosynthesis is coupled with the production of the safeguard molecule pentostatin. Cell Chemical Biology 24, 1479–1489.

Yang S, Jin L, Ren X, Lu J, Meng Q. 2014 – Optimization of fermentation process of *Cordyceps militaris* and antitumor activities of polysaccharides in vitro. Journal of Food and Drug Analysis 22, 468–476.

Zarei O, Dastmalchi S, Hamzeh-Mivehroud M. 2016 – A simple and rapid protocol for producing yeast extract from *Saccharomyces cerevisiae* suitable for preparing bacterial culture media. Iranian Journal of Pharmaceutical Research 15, 907.

Zeng J, Zhou Y, Lyu M, Huang X et al. 2024 – *Cordyceps militaris*: A novel mushroom platform for metabolic engineering. Biotechnology Advances 74, 108396.

Zhang WX, Zhong JJ. 2013 – Oxygen limitation improves ganoderic acid biosynthesis in submerged cultivation of *Ganoderma lucidum*. Biotechnology and Bioprocess Engineering 18, 972–980.

Zhu K, Ruan H, Wu T, Zhang H et al. 2024 – Exploiting the roles of nitrogen sources for HEA increment in *Cordyceps cicadae*. Frontiers in Microbiology 15, 1384027.

Supplementary Table S1. Analysis of variance (ANOVA)[a] of factors affecting the productivity of adenosine

Factor [b]	DOF	Sum of Squares	Variance	F–Ratio	Pure Sum	Percent P (%)	Confidence (%)	Significant level*
A	2	0.616	0.308	22.355	0.588	14.638	100.00	0.000
B	2	0.310	0.155	11.250	0.282	7.026	99.93	0.001
C	2	2.074	1.037	75.266	2.046	50.907	100.00	0.000
D	2	0.769	0.385	27.907	0.741	18.444	100.00	0.000
Other/Error	18	0.248	0.014			8.986		
Total	26	4.020	0.155			100.000		

$Y_{expected} = \overline{T} + (\overline{A}_{opt} - \overline{T}) + (\overline{B}_{opt} - \overline{T}) + (\overline{C}_{opt} - \overline{T}) + (\overline{D}_{opt} - \overline{T})$T

Note $Y_{expected}$, the expected value; $\overline{T}$, the grand average of performance; $\overline{A}_{opt}$, maximum average effect of factor A; $\overline{B}_{opt}$, maximum average effect of factor B; $\overline{C}_{opt}$, maximum average effect of factor C; $\overline{D}_{opt}$, maximum average effect of factor D.

[a] ANOVA was for the experiments shown in Table 4.2.

[b] A, Fungal strain; B, glucose concentration (g/L); C, yeast extract concentration (g/L); D, initial pH.

* Significant at $p < 0.01$