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Research Article

# การผลิตคอร์ไดเซปินและอะดีโนซีนอย่างยั่งยืนจากเห็ดถั่งเช่าสีทองโดยใช้ผลพลอยได้จากอุตสาหกรรมเกษตร

# Sustainable Production of Cordycepin and Adenosine from *Cordyceps militaris* Using Agro-Industrial Byproducts

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## บทคัดย่อ

ผลพลอยได้จากอุตสาหกรรมเกษตรเป็นทรัพยากรที่มีอยู่มากและต้นทุนต่ำ ซึ่งมีศักยภาพในการเปลี่ยนรูปทางชีวภาพไปเป็นสารออกฤทธิ์ทางชีวภาพที่มีมูลค่าสูง การศึกษานี้ประเมินการใช้เปลือกถั่วเหลือง (SB-H) ชานอ้อย (SC-B) และแกลบ (RH-K) เป็นวัสดุเพาะเลี้ยงเห็ดถั่งเช่าสีทอง (*Cordyceps militaris*) ภายใต้สภาวะหมักในของแข็งโดยใช้ข้าวกล้องหอมมะลิเป็นกลุ่มควบคุม ผลการทดลองพบว่า อาหารเพาะเลี้ยงสูตรเปลือกถั่วเหลืองให้ผลผลิตชีวมวล 3.04 ± 0.06 กรัม ซึ่งไม่แตกต่างอย่างมีนัยสำคัญกับสูตรอาหารข้าวกล้องหอมมะลิ (3.07 ± 0.06 กรัม) แต่อย่างไรก็ตาม สูตรอาหารเพาะเลี้ยง

สูตรเปลือกถั่วเหลืองสามารถส่งเสริมการผลิตสารออกฤทธิ์ทางชีวภาพได้สูงกว่าอย่างมีนัยสำคัญ โดยมีคอร์ไดเซปิน 7.76 ± 0.26 มิลลิกรัม/กรัม น้ำหนักแห้ง (เพิ่มขึ้นร้อยละ 22.40 เมื่อเทียบกับกลุ่มควบคุม) และมีปริมาณอะดีโนซีน 0.47 ± 0.02 มิลลิกรัม/กรัม น้ำหนักแห้ง นอกจากนี้ การใช้เปลือกถั่วเหลืองยังช่วยลดต้นทุนการผลิตต่อกรัมของน้ำหนักแห้งได้ร้อยละ 7.54 การวิเคราะห์วัสดุเพาะฐานรองดอก พบว่ามีสารสำคัญตกค้างอยู่ในระดับสูง โดยวัสดุเพาะฐานรองดอกจากอาหารสูตรเปลือกถั่วเหลืองมีคอร์ไดเซปินตกค้างสูงสุด (6.83 ± 0.16 มิลลิกรัม/กรัม น้ำหนักแห้ง) ขณะที่วัสดุเพาะฐานรองดอกจากอาหารสูตรชานอ้อยมีอะดีโนซีนตกค้างสูงสุด (0.37 ± 0.02 มิลลิกรัม/กรัม น้ำหนักแห้ง) แสดงให้เห็นถึงศักยภาพในการนำวัสดุเพาะฐานรองดอกกลับมาใช้ประโยชน์ ผลการศึกษานี้ชี้ให้เห็นว่าผลพลอยได้จากอุตสาหกรรมเกษตรสามารถใช้เป็นวัสดุเพาะเลี้ยงทางเลือกที่ยั่งยืนและมีประสิทธิภาพในการผลิตเห็ดถั่งเช่าสีทอง ทั้งในด้านการสร้างมวลชีวภาพ การเพิ่มการผลิตสารออกฤทธิ์ และการสนับสนุนเศรษฐกิจหมุนเวียนในการเพาะเลี้ยงเห็ดสมุนไพร

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## Abstract

Agro-industrial byproducts are abundant, low-cost resources with strong potential for bioconversion into high-value bioactive compounds. This study explored the use of soybean hulls (SB-H), sugarcane bagasse (SC-B), and rice husk (RH-K) as alternative substrates for the cost-effective cultivation of *Cordyceps militaris* under solid-state fermentation, with jasmine brown rice (JBR) serving as the control. The results indicated that SB-H produced a biomass yield of 3.04 ± 0.06 g dry weight (DW), which was not significantly different from JBR (3.07 ± 0.06 g DW). However, SB-H significantly ($p \leq 0.05$) enhanced the production of bioactive compounds, yielding cordycepin at 7.76 ± 0.26 mg/g DW, representing a 22.40% increase over the control, and adenosine at 0.47 ± 0.02 mg/g DW. In addition, cultivation with SB-H reduced the production cost per gram of dry biomass by 7.54%. Analysis of spent mushroom substrates (SMS) revealed high levels of residual compounds, with SB-H containing the highest residual cordycepin content (6.83 ± 0.16 mg/g DW), while SC-B contained the highest residual adenosine content (0.37 ± 0.02 mg/g DW), highlighting their potential for secondary utilization. These findings demonstrate that agro-industrial byproducts, particularly soybean hulls, can serve as sustainable and efficient alternative substrates for the cultivation of *C. militaris*, offering comparable biomass yield, enhanced bioactive compound production, cost reduction, and support for circular economy practices in medicinal mushroom cultivation.

*Keywords:* agro-industrial byproducts; medicinal mushrooms; sustainability; bioactive compounds

## Introduction

The rapid expansion of global agricultural activities has led to the generation of massive amounts of agro-industrial byproducts. Despite their vast potential for reuse, a substantial portion of these byproducts remains either neglected or mismanaged, resulting in serious environmental consequences such

as soil degradation, water pollution, and increased greenhouse gas emissions (Priyanka et al., 2024) [1]. Numerous studies have emphasized the potential of agricultural residues as valuable resources rather than mere waste. These byproducts are typically rich in essential components such as carbohydrates, proteins, lignocellulosic fibers, minerals, and various bioactive compounds, including phenolics and flavonoids (Raţu et al., 2023) [2]. Owing to these valuable constituents, agro-waste serves as a promising resource for a wide range of biotechnological applications, particularly as substrates for mushroom cultivation.

*Cordyceps militaris* has emerged as one of the most promising medicinal mushrooms, drawing increasing scientific and commercial interest due to its remarkable pharmacological properties. Known commonly as the orange caterpillar fungus, *C. militaris* can be efficiently cultivated on both solid and liquid media using a wide variety of carbon and nitrogen sources, making it highly adaptable for biotechnological applications. *C. militaris* was proven to produce a broad spectrum of bioactive compounds, including polysaccharides, phenolics, proteoglycans, terpenoids, steroids, lectins, ergosterol, and nucleosides such as adenosine and cordycepin (3′-deoxyadenosine) (Ashraf et al., 2020) [3], (Elkhateeb et al., 2019) [4]. Among these, cordycepin stands out as the most significant due to its potent therapeutic and nutraceutical properties. It has been linked to a wide range of health benefits, including anti-diabetic, anti-hyperlipidemic, antifungal, anti-inflammatory, antioxidant, anti-aging, and anticancer effects.

Traditionally, *C. militaris* has been cultivated on a variety of solid media rich in carbon and nitrogen sources. Common substrates include silkworm pupae, edible insects (Trung et al., 2024) [5], and cereal grains such as brown rice, millet, sorghum, corn (Wen et al., 2014) [6], rice, wheat (Tao et al., 2020) [7], and other grains (Borde and Singh, 2023) [8], all of which support mycelial growth and the production of secondary metabolites. However, the increasing cost and demand for these cereal grains, many of which are essential staple foods for both humans and livestock, raise sustainability and food security concerns. Moreover, the use of animal-based substrates like silkworm pupae poses additional challenges, including dietary restrictions, potential allergenicity, scalability, consumer acceptance, regulatory challenges, and inconsistency in cultivation outcomes. As a result, there is a growing need to identify low-cost, plant-based, and sustainably sourced substrates that can support efficient and scalable cultivation. Such alternatives are not only key to reducing production costs but also vital for promoting circular bioeconomy practices in medicinal mushroom production.

Agro-industrial byproducts are increasingly being investigated as alternative substrates for mushroom cultivation due to their abundance, low cost, and favorable nutrient profiles. Previous studies have demonstrated the successful use of materials such as cottonseed shells, corn cob particles, Italian poplar sawdust, and spent substrates from *Flammulina velutipes* to enhance biomass production as well as the yields of cordycepin and adenosine in *C. militaris* (Lin et al., 2017) [9]. Borde and Singh (2023) [8] also reported that supplementing solid-state fermentation with a mixture of grains and sugarcane bagasse significantly enhanced cordycepin production in *C. militaris* fruiting bodies. While agro-industrial byproducts show considerable potential, their use as primary substrates for *C. militaris* cultivation remains relatively

underexplored. By repurposing agricultural waste, this approach not only helps reduce environmental burden and production costs but also contributes to the circular economy by aligning with principles of bioeconomy, resource efficiency, and green innovation, ultimately supporting sustainable and value-added mushroom production.

## Objectives

This study aims to assess the potential of agro-industrial byproducts as alternative substrates to promote fungal growth and enhance bioactive compound production in *C. militaris.*

## Methods

### 1. Chemicals and materials

All chemicals and solvents used in this study were of analytical reagent grade. Methanol (AR grade, 99.9%) and HPLC-grade methanol were used for the extraction and analysis procedures. Standards of cordycepin and adenosine were obtained from Sigma Chemical Corporation (Saint Louis, MO, USA). For *C. militaris* cultivation, jasmine brown rice (JBR) was purchased from local retailers in Nonthaburi Province, Thailand. Agro-industrial byproducts, including sugarcane bagasse (SC-B) and rice husk (RH-K), were obtained from an agricultural farm in Nonthaburi Province, whereas soybean hulls (SB-H) were procured from agricultural suppliers in Nakhon Pathom Province, Thailand.

### 2. Collection and preparation of substrates

All substrates were sorted to remove foreign matter and thoroughly washed three times with tap water. JBR was soaked in water for approximately 15 min to achieve a moisture content of 60–70%. After soaking, excess water was drained prior to use. Three agricultural residues, including SC-B, SB-H, and RH-K, were dried in a hot-air oven at 60 °C until their moisture content was reduced to below 15–20%. The dried materials were then chopped and ground using a household blender to obtain a particle size of no more than 5 mm. Subsequently, SC-B, SB-H, and RH-K were analyzed for carbon and nitrogen contents using a CHN Analyzer (FlashSmart, Thermo Scientific, Italy) to determine their C/N ratio, which were further evaluated in this study.

### 3. Preparation of *C. militaris* seed culture

A strain of *Cordyceps militaris* was obtained from Sunanta Farm in Nonthaburi Province, Thailand. Species identity was confirmed by DNA barcoding of the internal transcribed spacer (ITS) region using universal primers ITS1/ITS4, followed by BLAST analysis against the GenBank database (accession no. ON795947), which revealed >99% sequence similarity to reference sequences of *C. militaris*. The strain was subsequently cultured on potato dextrose agar (PDA; HiMedia Laboratories Pvt. Ltd., Mumbai, India) slants at 20 °C for 10 days to promote mycelial growth. The actively growing mycelium was then transferred to a liquid culture. A 250 mL flask containing 100 mL of fresh potato dextrose broth (PDB)

was inoculated with 2–3 pieces (~0.6 $cm^2$ each) of mycelial slant from the PDA culture. The flask was incubated at 25 °C with an agitation of 120 rpm. Maximum mycelial growth was observed after 7 days. The culture was maintained under routine conditions, and intact mycelium was obtained for subsequent experiments.

**4. Cultivation of *C. militaris***

The modified solid medium was prepared in 720 mL glass jars. In this study, three types of agro-industrial byproduct substrates, SC-B (5 g), SB-H (15 g), and RH-K (15 g), were each mixed with 30 mL of modified potato dextrose broth (MPDB). Because these residues exhibited lower bulk density compared to cereal grains, the quantities were adjusted to achieve an equivalent culture volume per bottle. The mixtures were transferred into the jars. Substrates containing dry material were packed into the jars and pressed evenly. JBR medium served as the control, consisting of 30 g of JBR and 30 mL of MPDB. MPDB was prepared by blending three whole eggs with shells (approximately 180–190 g total weight) with 5 g of glucose and 1 L of distilled water. The resulting mixture was filtered before use. All media were prepared in triplicate. The assembled jars were sterilized at 121 °C for 30 min and then cooled under sterile airflow. Each sterilized jar was inoculated with 5 mL of liquid mycelial culture. Inoculated jars were incubated in the dark at 22 °C with 60–70% relative humidity for two weeks to allow for mycelial colonization. Following this, the cultures were transferred to an environment maintained at 24 °C and continuously exposed to fluorescent warm light at an intensity of 500 lux to induce stroma development. The number of days until primordia formation was recorded for each substrate. After 56 days of cultivation, the mature fruiting bodies were harvested, and their average lengths were measured. Dry weights were determined after dehydration at 60 °C for 6 hours (fruiting bodies) and 12 hours (spent mushroom substrates, SMS). Both the fruiting bodies and SMS, consisting of residual mycelium and remaining medium, were collected for further analysis.

**5. Extraction of bioactive compounds**

The dried samples were extracted using a solid–liquid extraction method with minor modifications based on the procedure by Pintathong et al. (2021) [10]. Briefly, 2 g of each sample was mixed with 10 mL of 60% (v/v) methanol at a 1:10 (w/v) ratio in clean volumetric flasks. The flasks were tightly sealed and shaken at 125 rpm at ambient temperature for 3 hours. To enhance extraction efficiency, the mixtures were then subjected to ultrasonication at 35 kHz (320 W) at 30 °C for 25 min. Following sonication, the mixtures were filtered under vacuum using Whatman No. 42 filter paper to remove solid residues. The filtrates were subsequently centrifuged at 2,810×g for 15 min. The resulting supernatants were filtered through a 0.22 µm nylon membrane and stored at 4 °C for subsequent quantification of cordycepin and adenosine.

**6. Quantification of bioactive compounds**

Quantitative analysis was performed using a high-performance liquid chromatography (HPLC) system (Agilent 1200 Series, Agilent Technologies, USA). Chromatographic separation was performed using reversed-phase C18 column (ZORBAX Eclipse XDB-C18, 4.6 mm × 150 mm, 5 µm; Agilent

Technologies, Santa Clara, CA, USA) maintained at 30 °C. An aliquot of 20 µL from each sample was injected into the system, and detection was conducted at 260 nm using a UV–Visible detector. A mobile phase of 20% methanol (v/v) was employed at a flow rate of 1 mL/min. Calibration curves covering the range of 0.2–1.0 mg/mL were generated for the quantification of cordycepin and adenosine, and concentrations were expressed as milligrams per gram of dry weight (mg/g DW).

**7. Statistical analysis**

A single-factor experimental design was employed to evaluate the effects of different substrate types. All treatments were conducted in triplicate, and results were reported as mean values with standard deviations (SD). Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS), version 28. A one-way analysis of variance (ANOVA) was conducted to determine statistically significant differences among treatments at a 95% confidence level ($p \leq 0.05$). When significant differences were identified, the Tukey–Kramer multiple comparison test was applied to assess pairwise differences between groups at the same confidence level.

## Results

**1. Morphological characteristics and mycelial growth**

In this study, three agro-industrial byproducts were used as alternative solid substrates for the cultivation of *C. militaris*. SC-B, derived from sugar production, had a C/N ratio of 67:1; RH-K, obtained from rice milling, exhibited a C/N ratio of 45:1; and SB-H, a byproduct of vegetable oil processing, showed a lower C/N ratio of 23:1. For comparison, the control substrate, JBR, had a C/N ratio of 28:1. However, preliminary observations indicated that these materials had a low bulk density, which could affect substrate compaction and fungal colonization compared to cereal grains commonly used in traditional cultivation. To address this issue, the quantities of the materials were adjusted accordingly to support aerobic growth conditions by providing sufficient space and surface area for mycelial development.

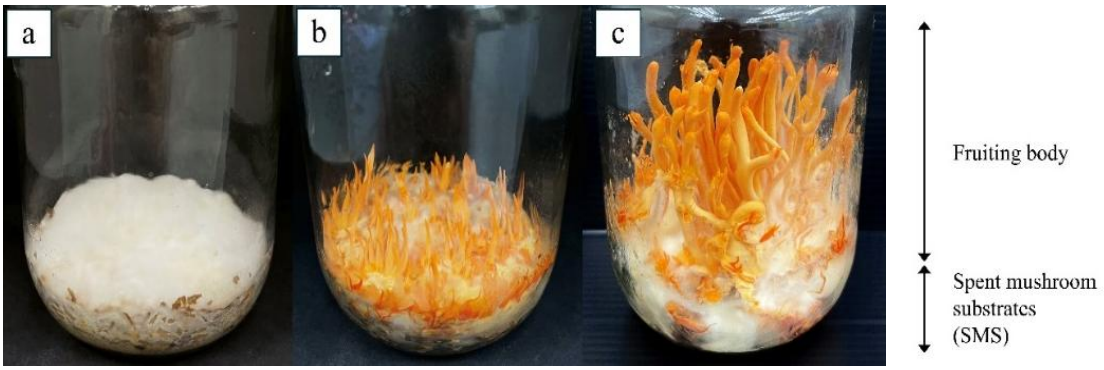


**Figure 1** Developmental stages of *C. militaris* cultivated on a solid substrate: (a) complete colonization of the mycelial mat across the medium surface, (b) initiation of primordia formation, and (c) fruiting body development after 56 days of inoculation under solid-state fermentation treatments.

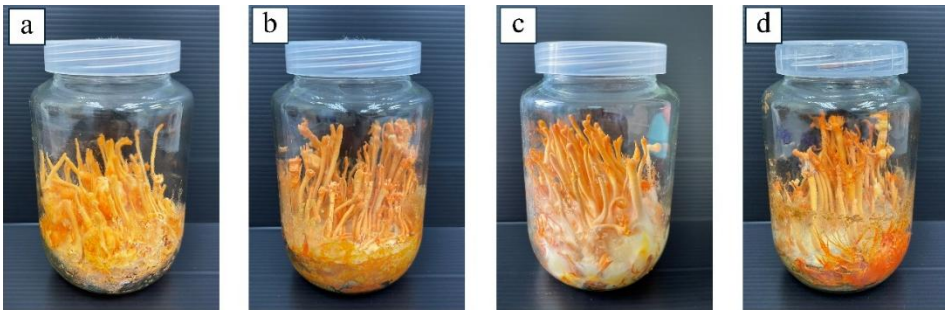


**Figure 2** Morphology of *C. militaris* cultivated on solid media formulated with different agro-industrial byproducts: (a) jasmine brown rice (JBR), (b) sugarcane bagasse (SC-B), (c) soybean hulls (SB-H), and (d) rice husk (RH-K).

The results showed that uniform white mycelial growth covered the substrates within 10 days after inoculation, except for SC-B, which exhibited delayed colonization. Complete colonization of the substrate surface by the mycelium was observed across all three agro-industrial byproduct substrates (Figure 1a), and the mycelium began to turn orange–yellow under warm light stimulation after two weeks of cultivation. Note that the mycelium darkened in color and increased in thickness over time. Primordia formation was observed on all substrates between days 16 and 17 after inoculation (Table 1). Most specimens developed club-shaped, slender fruiting bodies growing in dense clusters. The stipes were straight or slightly curved and exhibited vibrant orange–yellow pigmentation.

The fruiting bodies were harvested after 56 days. The morphology of the fruiting bodies on each substrate at harvest is shown in Figure 2. Variations in both the number and size distribution of fruiting bodies were evident among the different substrates. JBR produced the highest number of small fruiting bodies (1–3 cm: 33.00 ± 3.74), whereas SB-H and RH-K yielded a higher proportion of medium to large fruiting bodies, particularly in the 4–6 cm and 7–9 cm ranges. Notably, SB-H produced the greatest number of large fruiting bodies (7–9 cm: 8.67 ± 0.94), followed by SC-B (7.33 ± 1.25), suggesting that agro-industrial byproduct substrates may support enhanced elongation of fruiting bodies compared to JBR (Table 1). These findings indicate that agro-industrial byproducts, particularly SB-H and RH-K, can serve as viable alternative substrates for *C. militaris* cultivation without compromising key morphological and developmental traits. Moreover, their ability to support both mycelial proliferation and fruiting body formation further underscores their potential as effective cultivation substrates.

**2. Biomass yields of *C. militaris***

The biomass yields of *C. militaris* fruiting bodies and spent mushroom substrate (SMS) varied significantly among the different agro-industrial byproduct substrates used (Table 2). Among all treatments, SB-H yielded 3.04 ± 0.06 g dry weight (DW) of biomass, which was not significantly different from JBR (3.07 ± 0.06 g DW). In contrast, sugarcane bagasse (SC-B) and rice husk (RH-K) showed significantly lower

yields at 2.61 ± 0.08 g DW and 2.77 ± 0.13 g DW, respectively. This trend underscores the importance of substrate nutrient availability and structural characteristics in influencing fungal growth.

**Table 1** Mycelial growth and fruiting body development of *C. militaris* cultivated on agro-industrial byproducts.

| Substrates | Primordia initiation | | | Number of fruiting bodies | | |
|---|---|---|---|---|---|---|
| | | | | Length (cm) | | |
| | Mycelial density (7 days) | Fully colonized substrates (days) | Primordium formation (days) | 1-3 | 4-6 | 7-9 |
| JBR | ++++ | 10 | 16 | 33.00 ± 3.74$^{a}$ | 30.67 ± 3.30$^{a}$ | 3.33 ± 0.94$^{b}$ |
| SC-B | +++ | 11 | 17 | 24.67 ± 3.68$^{a}$ | 34.67 ± 3.40$^{a}$ | 7.33 ± 1.25$^{a}$ |
| SB-H | ++++ | 10 | 16 | 14.00 ± 1.41$^{b}$ | 40.00 ± 2.83$^{a}$ | 8.67 ± 0.94$^{a}$ |
| RH-K | +++ | 10 | 17 | 15.33 ± 2.05$^{b}$ | 33.00 ± 2.83$^{a}$ | 6.33 ± 0.94$^{ab}$ |

**Note:** Results are presented as mean ± S.D. from three independent replicates. Significant differences between groups were determined at the 95% confidence level ($p \leq 0.05$) and are indicated by different letters (a, b). Plus symbols (+) denote relative mycelial density after 7 days of incubation: + indicates sparse, ++ moderate, +++ dense, and ++++ very dense mycelial growth.

Following the harvest of fruiting bodies, the spent mushroom substrate (SMS), consisting of residual mycelium and remaining solid media, was collected from each cultivation treatment. The biomass of the SMS differed significantly among treatments. The highest SMS yield was observed in the JBR treatment (19.61 ± 0.15 g DW), while SB-H and RH-K exhibited moderate yields (14.77 ± 0.06 g DW and 14.54 ± 0.07 g DW, respectively). In contrast, SC-B showed the lowest residual biomass yield at 5.52 ± 0.07 g DW (Table 2). These variations are partly attributable to the initial density and physical mass of the substrates. JBR, being denser than the fibrous agro-industrial byproducts, provided a greater mass of starting material per volume, resulting in a higher residual biomass after fungal cultivation. In general, the cultivation of *C. militaris* produces SMS that is approximately 3–7 times greater in mass than the harvested fruiting bodies (Pintathong et al., 2021) [10].

**3. Bioactive compounds content in the fruiting body**

Substrate composition has a profound impact on the biosynthesis of bioactive compounds in *C. militaris* (Jędrejko et al., 2022) [11], particularly cordycepin (3′-deoxyadenosine, a therapeutic nucleoside analog) and adenosine (a key endogenous nucleoside) (Jędrejko et al., 2021) [12], both well known for their pharmacological properties. In this study, substantial variations in the concentrations of these compounds in the fruiting bodies were observed depending on the substrate used. The concentrations were assessed across solid media incorporating different agro-industrial byproducts, SC-B, SB-H, and RH-K, with JBR serving as the control.

**Table 2** Biomass yields of fruiting bodies and spent mushroom substrates (SMS) on different agro-industrial byproducts.

| Substrates | Biomass yields of *C. militaris* (g DW) | |
|---|---|---|
| | Fruiting body | SMS |
| JBR | 3.07 ± 0.06[a] | 19.61 ± 0.15[a] |
| SC-B | 2.61 ± 0.08[b] | 5.52 ± 0.07[c] |
| SB-H | 3.04 ± 0.06[a] | 14.77 ± 0.06[b] |
| RH-K | 2.77 ± 0.13[b] | 14.54 ± 0.07[b] |

**Note:** Results are expressed as mean ± S.D. from three independent replicates. Significant differences between groups were determined at a 95% confidence level ($p \leq 0.05$) and are indicated by different letters (a, b, c).

Cordycepin content ranged from 5.94 ± 0.29 to 7.76 ± 0.26 mg/g DW. As shown in Figure 3a, the type of substrate significantly affected cordycepin accumulation in the fruiting bodies. The highest level, 7.76 ± 0.26 mg/g DW, was achieved with the SB-H substrate. This represented a 22.40% increase compared to the control (JBR, 6.34 ± 0.20 mg/g DW), followed by RH-K (6.75 ± 0.10 mg/g DW) and SC-B (5.94 ± 0.29 mg/g DW). Furthermore, the cordycepin yield obtained with SB-H was approximately 38.08% higher than the value of 5.62 mg/g previously reported for brown rice substrate by Wen et al. (2014) [6].

Adenosine, a primary metabolite involved in cellular energy metabolism and nucleic acid biosynthesis (Dunn and Grider, 2023) [13], exhibited a similar trend in production across the different treatments. As shown in Figure 3b, under solid-state fermentation, the SB-H substrate treatment yielded the highest adenosine content in the fruiting bodies (0.47 ± 0.02 mg/g DW). However, this increase was not statistically significant when compared to the control treatment with JBR (0.41 ± 0.01 mg/g DW). The lowest adenosine content was observed in the RH-K treatment (0.35 ± 0.01 mg/g DW). Interestingly, in all treatments, cordycepin levels exceeded those of adenosine, suggesting that a substantial portion of adenosine may have been utilized as a precursor in cordycepin biosynthesis (Borde and Singh, 2023) [8], (Zhang et al., 2024) [14], (Pang et al., 2018) [15].

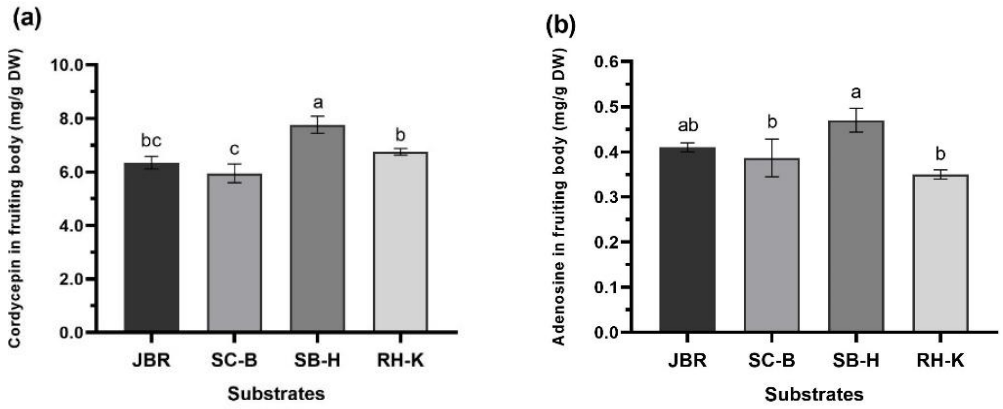


**Figure 3** Comparison of bioactive compound contents in *C. militaris* fruiting bodies cultivated on different substrates: (a) cordycepin and (b) adenosine. Error bars show SD from three replicates. Different letters indicate significant differences at $p \leq 0.05$.

**4. Bioactive compounds content in spent mushroom substrates**

At the end of the cultivation process, following the harvest of the fruiting bodies, a substantial amount of residual solid medium and mycelial biomass remained. Rather than discarding this biomass as waste, it was systematically collected and analyzed to assess its potential as a source of bioactive compounds. As shown in Figure 4, residual cordycepin content was highest in SB-H (6.83 ± 0.16 mg/g DW), with SC-B ranking second (6.20 ± 0.28 mg/g DW), highlighting the capacity of these substrates not only to promote initial metabolite synthesis but also to preserve significant amounts of bioactive compounds following the harvest of the mushroom. This finding aligns with previous research indicating that nutrient-rich or fibrous substrates can enhance and prolong metabolite accumulation during solid-state fermentation (Lin et al., 2017) [9], (Gregori, 2014) [16].

Interestingly, SC-B also showed the highest residual adenosine content (0.37 ± 0.02 mg/g DW), further supporting the hypothesis that the chemical structure and mineral composition of bagasse facilitate both growth and metabolite retention. In contrast, SMS derived from JBR and RH-K exhibited comparatively lower levels of cordycepin (4.34 ± 0.19 and 3.24 ± 0.14 mg/g DW, respectively), and adenosine (0.23 ± 0.01 and 0.21 ± 0.02 mg/g DW, respectively). Note that the persistence of high cordycepin levels in SB-H and SC-B implies that a significant fraction of the bioactive compounds remains unextracted or immobilized within the mycelial matrix and substrate after fruiting body development.

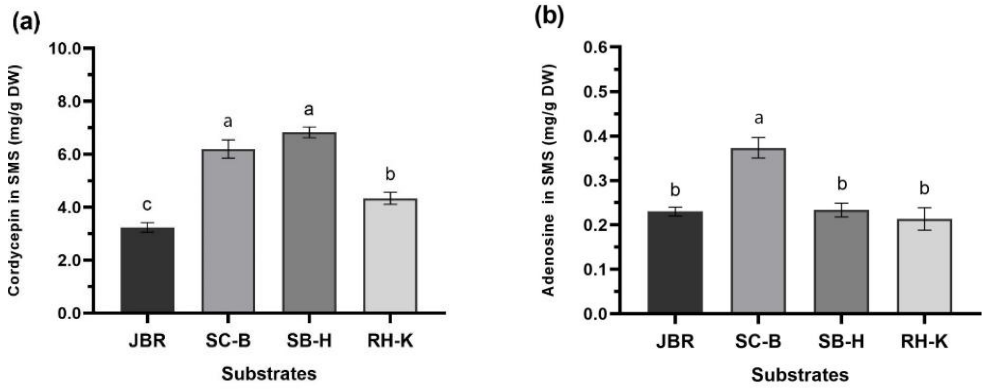


**Figure 4** Comparison of bioactive compound contents in spent mushroom substrates (SMS) derived from different cultivation substrates: (a) cordycepin and (b) adenosine. Error bars show SD from three replicates. Different letters indicate significant differences at $p \leq 0.05$.

## Conclusions and Discussion

The cultivation approach adopted in this study was designed as a sustainable alternative to conventional methods that commonly rely on insects or cereal grains (Wen et al., 2014) [6]. By investigating the use of agro-industrial byproducts as alternative substrates for *C. militaris* cultivation, the study aimed to enhance both fungal growth and the production of key bioactive compounds. The results clearly demonstrated that substrate composition significantly influenced biomass yield, as well as cordycepin and adenosine concentrations in both the fruiting bodies and spent mushroom substrates (SMS).

The enhanced growth of *C. militaris* using SB-H (Tables 1 and 2) may be attributed to a more favorable carbon-to-nitrogen (C/N) ratio and the presence of micronutrients that support fungal metabolism (Raethong et al., 2019) [17]. While JBR, used as the control, primarily provides starch as a readily accessible carbohydrate source, SB-H contributes higher nitrogen and a moderate level of fiber, ensuring a more sustainable growth profile through a balanced carbon–nitrogen supply. In contrast, SC-B and RH-K are rich in lignocellulose, with high C/N ratios and limited digestibility, which may hinder fungal colonization and enzymatic degradation. Previous studies have shown that *C. militaris* has limited ligninolytic activity and performs best on substrates with lower lignin content and balanced C/N ratios (Lin et al., 2017) [9]. As *C. militaris* is not a wood-decay fungus, it has limited enzymatic capacity to degrade lignin or crystalline cellulose (Zou et al., 2021) [18].

Interestingly, SB-H markedly enhanced the production of bioactive compounds in the fruiting bodies, surpassing JBR and other substrates (Figure 3). The superior performance of SB-H may be attributed to its relatively high nitrogen content, which is essential for the biosynthesis of nucleoside analogs such as cordycepin, as nitrogen supports the formation of purine backbones (Kontogiannatos et al., 2021) [19]. SB-H typically contains approximately 10–13% crude protein (Tokach et al., 2008) [20], providing a

substantial source of amino acids and adenine precursors that can enhance cordycepin production (Chang et al., 2024) [21]. Additionally, the partial availability of fermentable hemicellulose and the ability of soybean hulls to improve the physical structure of the substrate may facilitate better aeration and support mycelial growth (Shrestha et al., 2012) [22]. In contrast, JBR, though rich in starch, yielded lower cordycepin, suggesting that carbon-rich substrates alone are insufficient for optimal secondary metabolite synthesis. Previous studies reported that a C/N ratio of 12.7:1 was optimal for cordycepin production (Raethong et al., 2019) [17]. In this study, the substrates exhibited higher C/N ratios (SB-H 23:1, JBR 28:1, RH-K 45:1, SC-B 67:1), all above the reported optimum. The relative closeness of SB-H to the optimal ratio corresponded with its highest cordycepin yield, followed by JBR, RH-K, and SC-B, confirming that nitrogen availability strongly influenced metabolite accumulation. These findings align with the work of Kontogiannatos et al. (2021) [19], who emphasized the importance of balanced C/N ratios for optimizing cordycepin yields. A sufficient protein supply enhances production by providing essential precursors, such as adenine. These results are particularly noteworthy given that previous studies have reported much lower cordycepin concentrations in fruiting bodies cultivated on a mixture of cereal grains and sugarcane bagasse (1.93 mg/g DW) (Borde and Singh, 2023) [8]. Additionally, Wen et al. (2014) [6] reported that *C. militaris* grown on rice or cereal grains generally produces cordycepin in the range of 2.59–5.62 mg/g DW when extracted with methanol, which remains lower than the levels observed in this study. Furthermore, Phoungthong et al. (2022) [23] reported an adenosine content of 0.45 mg/g in fruiting bodies cultivated on riceberry, which is slightly lower than that found in our study. However, Wen et al. (2014) [6] reported higher adenosine levels of 0.61 mg/g DW and 0.83 mg/g DW in fruiting bodies grown on brown rice and wheat, respectively. Similarly, Borde and Singh, (2023) [8] reported an adenosine content of 3.392 mg/g, when using a mixed grain substrate composed of rice, wheat, jowar, and sugarcane bagasse. These findings contrast with those of our study and suggest that mixed grain-based substrates may promote greater adenosine accumulation compared to single-substrate cultivation. However, it is important to recognize that the levels of bioactive compounds can be influenced by a wide range of variables, including substrate type, fungal strain genetics, inoculum preparation, environmental conditions, air exchange and humidity, lighting, cultivation duration, and the methodologies used for extraction and analysis. These factors can significantly affect outcomes and often complicate direct comparisons across studies (Kontogiannatos et al., 2021) [19], (Shrestha et al., 2012) [22].

Analysis of SMS revealed substantial residual levels of bioactive compounds, particularly cordycepin and adenosine (Figure 4). The notably high cordycepin content detected in SB-H and SC-B indicates that a considerable proportion of these metabolites remains either unextracted or immobilized within the mycelial matrix and substrate even after fruiting body development. This observation is consistent with the findings of Pintathong et al. (2021) [10], who reported cordycepin concentrations of 0.78–1.09 mg/g in solid-based residues from *C. militaris* cultivation on different cereal substrates, values lower than those found in our study, suggesting that substrate composition may play an important role in metabolite retention. Such findings highlight the potential of SMS as a valuable secondary resource,

supporting diverse valorization pathways, including the recovery of residual bioactive compounds for pharmaceutical and cosmeceutical applications (Pintathong et al., 2021) [10], incorporation into animal feed to enhance nutritional quality, and utilization in biostimulants and soil fertilizers (Ma et al., 2025) [24]. These strategies not only reduce biowaste but also enhance resource efficiency, thereby reinforcing the sustainability and circularity of *C. militaris* production systems.

In conclusion, soybean hulls (SB-H) represent a promising substrate for the cultivation of *C. militaris*, yielding biomass comparable to jasmine brown rice (JBR) while markedly enhancing bioactive compound production and lowering production costs. As an abundant agro-industrial byproduct, SB-H offers a cost-effective and readily available alternative to conventional cereal grains, while simultaneously promoting sustainable practices in line with circular bioeconomy principles. Economically, replacing JBR with SB-H reduced production costs by approximately 7.54% per gram of dry biomass. While this reduction may seem relatively small, it becomes highly significant when scaled to industrial production. Furthermore, the substantial residual bioactive compounds retained in spent mushroom substrates underscore opportunities for secondary valorization, thereby extending value creation beyond the primary harvest and reinforcing the sustainability of *C. militaris* production systems. However, scaling up the use of agro-industrial byproducts for commercial applications requires careful consideration of key factors, including the costs and logistics of sourcing and processing, nutritional composition, potential contamination risks, seasonal availability, and quality consistency, all of which can critically influence fungal growth and metabolite biosynthesis.

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## References

[1] Priyanka, G., Singiri, J. R., Adler-Agmon, Z., Sannidhi, S., Daida, S., Novoplansky, N., and Grafi, G. (2024). Detailed analysis of agro-industrial byproducts/wastes to enable efficient sorting for various agro-industrial applications. *Bioresources and Bioprocessing*, *11*(1), Article 45.

[2] Rațu, R. N., Veleșcu, I. D., Stoica, F., Usturoi, A., Arsenoaia, V. N., Crivei, I. C., Postolache, A. N., Lipșa, F. D., Filipov, F., Florea, A. M., Chițea, M. A., and Brumă, I. S. (2023). Application of agri-food by-products in the food industry. *Agriculture*, *13*(8), Article 1559.

[3] Ashraf, S. A., Elkhalifa, A. E. O., Siddiqui, A. J., Patel, M., Awadelkareem, A. M., Snoussi, M., Ashraf, M. S., Adnan, M., and Hadi, S. (2020). Cordycepin for health and wellbeing: A potent bioactive metabolite of an entomopathogenic *cordyceps* medicinal fungus and its nutraceutical and therapeutic potential. *Molecules*, *25*(12), Article 2735.

[4] Elkhateeb, W., Daba, G., Thomas, P., and Wen, T.-C. (2019). Medicinal mushrooms as a new source of natural therapeutic bioactive compounds. *Egyptian Pharmaceutical Journal*, *18*(2), 88-101.

[5] Trung, N. Q., Dat, N. T., Anh, H. N., Tung, Q. N., Nguyen, V. T. H., Van, H. N. B., Van, N. M. N., and Minh, T. N. (2024). Substrate influence on enzymatic activity in *Cordyceps militaris* for health applications. *Chemistry*, *6*, 517-530.

[6] Wen, T.-C., Li, G. R., Kang, J.-C., Kang, C., and Hyde, K. (2014). Optimization of solid-state fermentation for fruiting body growth and cordycepin production by *Cordyceps militaris*. *Chiang Mai Journal of Science*, *41*, 858-872.

[7] Tao, S. X., Xue, D., Lu, Z. H., and Huang, H. L. (2020). Effects of substrates on the production of fruiting bodies and the bioactive components by different *Cordyceps militaris* strains (Ascomycetes). *International Journal of Medicinal Mushrooms*, *22*(1), 55-63.

[8] Borde, M., and Singh, S. K. (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.

[9] Lin, Q., Long, L., Wu, L., Zhang, F., Wu, S., Zhang, W., and Sun, X. (2017). Evaluation of different agricultural wastes for the production of fruiting bodies and bioactive compounds by medicinal mushroom *Cordyceps militaris*. *Journal of the Science of Food and Agriculture*, *97*(10), 3476-3480.

[10] Pintathong, P., Chomnunti, P., Sangthong, S., Jirarat, A., and Chaiwut, P. (2021). The feasibility of utilizing cultured *Cordyceps militaris* residues in cosmetics: Biological activity assessment of their crude extracts. *Journal of Fungi (Basel)*, *7*(11), Article 973.

[11] Jędrejko, K., Kała, K., Sułkowska-Ziaja, K., Krakowska, A., Zięba, P., Marzec, K., Szewczyk, A., Sękara, A., Pytko-Polończyk, J., and Muszyńska, B. (2022). *Cordyceps militaris*-fruiting bodies, mycelium, and supplements: Valuable component of daily diet. *Antioxidants* (*Basel*), *11*(10), Article 1861.

[12] Jędrejko, K. J., Lazur, J., and Muszyńska, B. (2021). *Cordyceps militaris*: An overview of its chemical constituents in relation to biological activity. *Foods*, *10*(11), Article 2634.

[13] Dunn, J., and Grider, M. H. (2023). *Physiology, adenosine triphosphate*. Treasure Island (FL): StatPearls Publishing.

[14] Zhang, H., Yang, J., Luo, S., Liu, L., Yang, G., Gao, B., Fan, H., Deng, L., and Yang, M. (2024). A novel complementary pathway of cordycepin biosynthesis in *Cordyceps militaris*. *International Microbiology, 27*(4), 1009-1021.

[15] Pang, F., Wang, L., Jin, Y., Guo, L., Song, L., Liu, G., and Feng, C. (2018). Transcriptome analysis of *Paecilomyces* hepiali at different growth stages and culture additives to reveal putative genes in cordycepin biosynthesis. *Genomics*, *110*(3), 162-170.

[16] Gregori, A., (2014). Cordycepin production by *Cordyceps militaris* cultivation on spent brewery grains. *Acta Biologica Slovenica*, *57*(2), 45-52.

[17] Raethong, N., Wang, H., Nielsen, J., and Vongsangnak, W. (2019). Optimizing cultivation of *Cordyceps militaris* for fast growth and cordycepin overproduction using rational design of synthetic media. *Computational and Structural Biotechnology Journal*, *18,* 1-8.

[18] Zou, G., Li, B., Wang, Y., Yin, X., Gong, M., Shang, J., Wei, Y., Li, X., and Bao, D. (2021). Efficient conversion of spent mushroom substrate into high value-added anticancer drug pentostatin with engineered *Cordyceps militaris. Green Chemistry*, *23*(24), 10030-10038.

[19] Kontogiannatos, D., Koutrotsios, G., Xekalaki, S., and Zervakis, G. I. (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 (Basel)*, *7*(11), Article 986.

[20] Barbosa, F. F., Tokach, M. D., DeRouchey, J. M., Goodband, R. D., Nelssen, J. L., and Dritz, S. S. (2008). Variation in chemical composition of soybean hulls. *Kansas Agricultural Experiment Station Research Reports*, *0*(10). https://doi.org/10.4148/2378-5977.7001

[21] Chang, Y., Liu, X., Jiao, Y., and Zheng, X. (2024). Improved cordycepin production by *Cordyceps militaris* using corn steep liquor hydrolysate as an alternative protein nitrogen source. *Foods*, *13*(15), Article 813.

[22] Shrestha, B., Zhang, W., Zhang, Y., and Liu, X. (2012). The medicinal fungus *Cordyceps militaris*: Research and development. *Mycological Progress*, *11*(3), 599-614.

[23] Phoungthong, K., Aiphuk, W., Maneerat, T., Suwunwong, T., Choto, P., and Chomnunti, P. (2022). Utilization of corncob biochar in cultivation media for cordycepin production and biomass of *Cordyceps militaris*. *Sustainability*, *14*(15), Article 9362.

[24] Ma, X., Yan, S., and Wang, M. (2025). Spent mushroom substrate: A review on present and future of green applications. *Journal of Environmental Management*, *373*, Article 123970.