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A Study on Culture Condition Optimization and Bioactive Evaluation of *Cordyceps militaris* for Maximizing Cordycepin and Fruiting Body Output**Rohit Rawat¹, Amita Gupta², Nidhi Tripathi³**¹ PhD Scholar, Department of Biochemistry, Mansarovar Global University, Bhopal, India.² Department of Biochemistry, Mansarovar Global University, Bhopal, India.³ Department of Biotechnology, Career College, Bhopal, India.**Email: rohitmycology@gmail.com****Article Information**

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compounds**ABSTRACT**

Cordyceps militaris, a valued entomopathogenic fungus, is known for its ability to biosynthesize bioactive compounds, particularly cordycepin (3'-deoxyadenosine), which exhibits potent antimicrobial, anticancer, and immunomodulatory properties. The present study aimed to optimize environmental and nutritional culture conditions to enhance both the biomass and cordycepin yield, alongside evaluating the phytochemical and antibacterial properties of solvent extracts from *C. militaris* fruiting bodies. The mother culture was maintained on Potato Dextrose Agar (PDA), and seed inoculum was propagated in Yeast Potato Dextrose Broth (YPDB). Parameters such as temperature (10–30 °C), pH (4–9), relative humidity (50–90%), carbon and nitrogen sources, and LED light spectra were systematically varied. Optimal conditions were found at 20 °C, pH 6, and 80% relative humidity, with glucose, peptone, and yeast extract proving to be the most effective nutrient sources. White LED light further enhanced cordycepin synthesis. Biomass and cordycepin content were quantified through dry weight analysis and High-Performance Liquid Chromatography (HPLC), respectively. Solvent extraction using petroleum ether, acetone, ethanol, and distilled water was performed on dried fruiting bodies. Acetone extracts exhibited the richest phytochemical profiles and strongest antibacterial activity, with inhibition zones reaching up to 32 mm against *Staphylococcus epidermidis*. Thin-layer chromatography (TLC) validated the presence of flavonoids, phenolics, alkaloids, and other secondary metabolites. Antibacterial efficacy was evaluated through disc diffusion, agar well methods, and MIC/ MBC determination. The study concludes that standardized cultivation conditions and solvent extraction strategies significantly improve cordycepin production and biological activity. Future improvements may include media optimization via statistical modeling, genetic engineering, and omics-based approaches to further enhance yield and therapeutic efficacy of *C. militaris*.

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1. INTRODUCTION:

In recent years, *Cordyceps militaris* has emerged as a focus of biotechnological interest due to its rich repertoire of pharmacologically active compounds, particularly cordycepin. This species, long prized in traditional East Asian medicine, is now recognized globally for its anti-tumor, immunomodulatory, and anti-inflammatory activities. Owing to increasing demand, there has been a notable shift from wild harvesting to controlled cultivation under optimized

environmental and nutritional conditions (Chou et al., 2024). Industrial-scale production is now driven by bioreactor-based systems and solid-state fermentation technologies, which enable better yield management and metabolite consistency. Moreover, modern approaches like statistical optimization, machine learning algorithms, and integrated omics have allowed researchers to refine substrate compositions and environmental parameters, significantly improving cordycepin output and mycelial biomass (Liu et al., 2023; Zhang et al., 2024). These advancements support sustainable commercial exploitation of *C. militaris* as a nutraceutical and therapeutic agent, particularly in the face of overexploitation of wild strains.

A key determinant of cordycepin biosynthesis lies in the composition and balance of the nutrient medium. Traditional studies have focused on isolated variables such as glucose concentration or nitrogen source; however, recent investigations have highlighted the synergistic roles of micronutrients, vitamins, and carbon–nitrogen ratios in modulating gene expression linked to secondary metabolite pathways (Tang et al., 2023). Advanced fermentation techniques now incorporate metabolic profiling and response surface methodology (RSM) to design customized media that stimulate higher yields of cordycepin without compromising fungal health.

Additionally, genetic insights into purine metabolism and AMP biosynthesis pathways have shed light on how specific nutrients can activate or suppress biosynthetic genes (Zhang et al., 2024). These findings not only inform cost-effective cultivation strategies but also enhance consistency and bioactivity in large-scale production systems, offering a platform for standardized pharmaceutical applications of *C. militaris* (Wang et al., 2022).

Beyond nutritional factors, environmental signals—particularly light quality and intensity—play a vital role in regulating fungal morphogenesis and secondary metabolite production. Recent transcriptomic analyses have revealed that exposure to specific wavelengths, such as white and red LED light, upregulates genes associated with cordycepin biosynthesis, fruiting body maturation, and photoreceptor-mediated pathways (Kim et al., 2023). These findings are reshaping cultivation strategies by enabling the manipulation of circadian rhythm-like responses to maximize productivity. However, scalability remains a significant challenge. While small-scale laboratory models offer insight into environmental sensitivities, they often fail to translate effectively into industrial settings. To bridge this gap, biotechnological innovations such as programmable photobioreactors

and AI-integrated monitoring systems are being employed to replicate optimal light and humidity conditions at scale (Xu et al., 2024). These dynamic systems not only enhance yield stability but also facilitate reproducibility, a key requirement for regulatory compliance in nutraceutical and pharmaceutical industries.

Despite substantial progress, existing literature still lacks a unified model that integrates nutrient dynamics, environmental stimuli, and biochemical output to optimize both fruiting body development and cordycepin biosynthesis in *Cordyceps militaris*. Most studies have examined isolated parameters, often under non-scalable laboratory conditions, leaving a critical gap in understanding how these variables interact synergistically under real-world constraints (Zheng et al., 2021). Furthermore, the comparative effectiveness of different carbon and nitrogen sources, humidity levels, and light spectra remains fragmented across studies.

Against this backdrop, the present investigation aims to provide a comprehensive evaluation of physical, nutritional, and environmental factors affecting the growth and bioactive metabolite production of *C. militaris*. By integrating phytochemical profiling, antibacterial assessment, and HPLC-based quantification of cordycepin under controlled experimental setups, this study seeks to define optimal culture conditions that can serve both research and industrial cultivation systems. The findings are expected to offer actionable insights into scalable, efficient, and pharmacologically potent production strategies for *C. militaris*-based therapeutic applications.

REVIEW OF LITERATURE:

1. Pharmacological Potential and Traditional Use of *Cordyceps militaris*:

Cordyceps militaris, a well-known entomopathogenic fungus, has long been used in traditional Chinese medicine as a tonic with multiple therapeutic effects including anti-aging, anti-fatigue, and respiratory support (Holliday & Cleaver, 2008). Its growing recognition as a natural immunomodulator, antioxidant, and antitumor agent has drawn considerable scientific interest (Khan et al., 2010; Mehra et al., 2017). Unlike the endangered *Cordyceps sinensis*, *C. militaris* can be mass-cultivated under laboratory conditions, making it more accessible for pharmaceutical exploitation (Xiao et al., 2009).

2. Cordycepin: Structure, Synthesis, and Bioactivity:

Cordycepin (3'-deoxyadenosine), the key bioactive compound in *C. militaris*, exhibits anticancer, antiviral, and anti-inflammatory activities by

interfering with RNA synthesis and signaling pathways (Yoo et al., 2004). Recent work by Zhang et al. (2024) discovered an additional biosynthetic pathway in high-yield strains involving ATP-mediated enhancement of cordycepin and cyclic AMP production. Genetic studies using CRISPR/Cas9 (Jin et al., 2020) have identified candidate genes for targeted overexpression to enhance cordycepin biosynthesis in fruiting bodies.

3. Role of Nutritional Factors:

Several studies emphasize the role of nutrient optimization in enhancing cordycepin output. Song et al. (2016) and Tang et al. (2023) highlighted the importance of carbon–nitrogen ratios, with glucose and organic nitrogen sources like yeast extract and peptone promoting mycelial biomass and metabolite synthesis. Advanced media formulations using response surface methodology (RSM) now integrate vitamins and trace minerals, offering more consistent yields across scales.

4. Environmental Parameters: Temperature, pH, Humidity, and Light:

Optimal growth conditions for *C. militaris* were recorded at 20–25°C, pH 6–7, and 70–80% humidity (Zhou et al., 2021). Kim et al. (2023) demonstrated that specific light wavelengths modulate gene expression associated with cordycepin biosynthesis, with white and red LEDs showing the most favorable effects. These environmental factors are not only critical for mycelial health but also regulate secondary metabolism through light-responsive transcription factors.

5. Technological Innovations in Large-scale Cultivation:

Recent breakthroughs in cultivation technologies include programmable photobioreactors and artificial intelligence (AI)-based feedback control systems (Xu et al., 2024). Machine learning models help predict optimal culture conditions by integrating omics data, environmental settings, and metabolite outcomes (Zheng et al., 2021). These methods reduce dependency on trial-and-error experiments and accelerate scalable production.

6. Phytochemical Profiling and Antibacterial Properties:

The bioactive profile of *C. militaris* extends beyond cordycepin, including steroids, terpenoids, flavonoids, and alkaloids (Shweta et al., 2023). Extraction with solvents like acetone has shown superior antibacterial activity, particularly against *Staphylococcus epidermidis* and *Yersinia enterocolitica* (Chou et al., 2024). TLC and HPLC-based fingerprinting have validated the phytochemical diversity under different extraction methods, reinforcing its pharmaceutical potential.

3. MATERIALS AND METHODS:

3.1 Source and Maintenance of Mother Culture:

The mother culture of *Cordyceps militaris* was procured from the **Directorate of Mushroom Research (DMR), Solan**, Himachal Pradesh, India. To ensure sustained viability and physiological stability, the culture was maintained on **Potato Dextrose Agar (PDA)** medium and incubated at **25 °C for 14 days** under aseptic conditions. PDA is a widely used medium for fungal growth due to its richness in carbohydrates and essential nutrients (Barros et al., 2007). The cultures were routinely examined for contamination, and mycelial integrity was preserved through periodic subculturing.

3.2 Culture Conditions and Inoculum Preparation:

3.2.1 Subculturing and Stock Maintenance

Fully colonized PDA cultures were stored at **4°C** as stock cultures. **Subculturing was done every two months** to ensure culture freshness and prevent degeneration. This practice ensures consistent inoculum performance and has been standard in fungal biotechnology labs (Masuda et al., 2006).

3.2.2 Seed Culture Development:

To prepare the seed inoculum, **0.5% Tween 80** was added to PDA slants to facilitate spore dispersion. A **4% spore suspension (~2.0 × 10⁹ spores/mL)** was aseptically transferred into seed broth for liquid culture development (Raethong et al., 2020).

3.2.3 Liquid Culture and Incubation:

Seed cultures were inoculated into **Yeast Potato Dextrose Broth (YPDB)** consisting of glucose (20 g/L), peptone (10 g/L), and yeast extract (5 g/L), with pH adjusted to **6.0** before sterilization. The cultures were incubated at **25 °C and shaken at 200 rpm** in 250 mL Erlenmeyer flasks containing 50 mL broth (Mao et al., 2004).

3.3 Biomass Estimation:

After incubation, mycelial biomass was collected by **centrifugation at 6000 rpm for 10 minutes**, washed with sterile distilled water, filtered using Whatman No. 1 filter paper, and **oven-dried at 45°C** to a constant weight. Dry weight was recorded as g/50 mL and used as the measure of fungal biomass (Zhang et al., 2024).

3.4 Optimization of Environmental and Nutritional Factors:

To determine the effect of growth conditions on biomass and cordycepin yield, various environmental and nutritional parameters were optimized.

3.4.1 Temperature, pH, and Humidity:

The influence of **temperature** was evaluated by incubating cultures at 10, 15, 20, 25, and 30 °C for 6 days. **pH** values ranging from 4 to 9 were adjusted prior to sterilization. **Humidity** levels of 50% to 90% were tested using controlled chambers. These parameters were based on prior optimization studies by Hung et al. (2009), which showed that cordycepin production is highly sensitive to such conditions.

3.4.2 Carbon and Nitrogen Source Optimization:

Six carbon sources (glucose, fructose, sucrose, maltose, lactose, starch) and six nitrogen sources (peptone, yeast extract, ammonium nitrate, ammonium sulfate, sodium nitrate, urea) were incorporated into the basal medium. Each treatment was inoculated with 4% seed culture and incubated at **20 °C, 200 rpm for 6 days**. Biomass and cordycepin concentrations were measured as endpoints (Mao et al., 2004; Bhandari et al., 2022).

3.5 Cordycepin Quantification via HPLC:

Quantification of **cordycepin (3'-deoxyadenosine)** was performed using **High-Performance Liquid Chromatography (HPLC)** following the method of Zhou et al. (2016). Dried biomass was extracted with methanol, sonicated for 30 minutes, filtered, and concentrated using rotary evaporation. Samples were reconstituted in a methanol-water mobile phase and injected into a **C18 reverse-phase HPLC column**. Cordycepin was detected at **254 nm**, and concentrations were determined using a calibration curve based on standard solutions.

3.6 Preparation of Fungal Powder and Extraction of Bioactives:

3.6.1 Drying and Powder Preparation:

Fruiting bodies of *Cordyceps militaris* were **oven-dried at 45°C for 24 hours**, then ground into a fine powder using a sterile grinder. The powder was stored in **airtight containers at 4°C** until extraction.

3.6.2 Soxhlet Extraction Using Various Solvents:

Bioactive compounds were extracted using a **Soxhlet apparatus** with solvents of increasing polarity: **petroleum ether, acetone, ethanol, and distilled water**. Each cycle was run until the solvent became colorless. Extracts were concentrated under reduced pressure, sterilized with UV light, and stored at 4°C (Chou et al., 2024; Shweta et al., 2023).

3.7 Phytochemical Screening:

Phytochemical analysis was conducted on each extract using standard **qualitative assays**. The presence of steroids, terpenoids, flavonoids, alkaloids, tannins, saponins, amino acids, phenolic compounds, cardiac glycosides, and volatile oils was detected using biochemical color reactions such as Dragendorff's test, Salkowski's test, and ferric

chloride test (Kokate, 2005). This method helps confirm the chemical nature of secondary metabolites relevant for pharmacological activity.

3.8 Antibacterial Activity Assay:

3.8.1 Disc Diffusion and Agar Well Methods:

The antibacterial efficacy of all extracts was tested against **Gram-positive bacteria** (*Staphylococcus aureus*, *Streptococcus pyogenes*) and **Gram-negative bacteria** (*Escherichia coli*, *Pseudomonas aeruginosa*). Extracts (100 mg/mL in DMSO) were impregnated onto sterile discs and placed on freshly seeded nutrient agar plates. Plates were incubated at 37°C for 24 hours. **Gentamycin (10 µg)** served as the positive control.

3.8.2 MIC and MBC Determination:

Minimum Inhibitory Concentration (MIC) and **Minimum Bactericidal Concentration (MBC)** were assessed by the broth microdilution technique in 96-well plates. Bacterial growth inhibition was monitored by optical density at **600 nm** and by colorimetric indicators. These methods were adapted from protocols by Shrestha et al. (2019) and comply with CLSI (2020) standards.

4. RESULTS

4.1 Effect of Temperature, pH, and Humidity on Growth and Cordycepin Content of *Cordyceps militaris*:

Environmental factors such as temperature, pH, and relative humidity were found to play a critical role in the mycelial growth and secondary metabolite production of *Cordyceps militaris*. Among the temperature ranges tested (10°C to 30°C), the fungus exhibited the highest biomass accumulation (1.21 g) and cordycepin production (5.62 mg) at 20°C. A gradual decline in both parameters was observed at 25°C and 30°C, indicating thermal sensitivity. Likewise, the maximum cordycepin content (6.23 mg) was recorded at pH 6, whereas mycelial biomass peaked at pH 7. Humidity also influenced fungal physiology, with 80% relative humidity supporting optimal growth (1.43 g) and cordycepin yield (5.72 mg). These results confirm that precise environmental control is essential for maximizing cordycepin production.

Table 1. Effect of Temperature, pH, and Humidity on Mycelial Biomass and Cordycepin Production in *Cordyceps militaris*

Parameter Type	Value	Dry Weight of Mycelia (g)	Cordycepin Content (mg)
Temperature	10°C	0.34	1.00
	15°C	0.64	3.22

	20°C	1.21	5.62
	25°C	0.93	4.54
	30°C	0.61	2.98
pH	4	0.98	4.33
	5	1.31	5.26
	6	1.42	6.23
	7	1.56	5.12
	8	0.94	2.99
	9	0.72	2.11
Humidity	50%	0.55	2.11
	60%	0.76	3.21
	70%	1.11	4.33
	80%	1.43	5.72
	90%	0.65	4.11

4.2 Effect of Carbon, Nitrogen, and Light Sources on *Cordyceps militaris*:

Different nutritional and photic conditions influenced the growth and cordycepin production significantly. Glucose was identified as the most suitable carbon source (5.77 mg cordycepin, 1.33 g biomass), followed by sucrose and fructose. Among nitrogen sources, organic ones like peptone and yeast extract were superior to inorganic salts. For lighting, white LED exposure resulted in the highest biomass and cordycepin content (5.21 mg), suggesting that broad-spectrum light enhances biosynthetic pathways.

Table 2. Effect of Carbon, Nitrogen, and Light Sources on Mycelial Biomass and Cordycepin Production in *Cordyceps militaris*

Parameter Type	Source	Dry Weight of Mycelia (g)	Cordycepin Content (mg)
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Carbon Source	Glucose	1.33	5.77
	Fructose	0.74	3.01
	Sucrose	0.91	4.32
	Maltose	0.83	3.55
	Lactose	0.51	2.43
Nitrogen Source	Starch	0.43	0.98
	Peptone	1.63	5.43
	Yeast extract	1.66	5.33
	Ammonium nitrate	0.95	4.22
	Ammonium sulfate	0.43	3.21
Light Source	Urea	0.64	2.32
	Sodium nitrate	0.23	2.10
	Pink LED	0.91	4.22
	Green LED	0.75	3.21
	Blue LED	0.55	2.67
	White LED	1.33	5.21

4.3 Chemical Composition of Different Extracts:

Phytochemical screening demonstrated that *C. militaris* contains a wide array of bioactive compounds, particularly in acetone and ethanol extracts. Steroids, terpenoids, flavonoids, alkaloids, and phenolic compounds were commonly detected. Water extract had limited composition, while acetone extract showed the most diverse chemical constituents.

Table 3. Phytochemical Screening of Various Solvent Extracts of *Cordyceps militaris*

S. No.	Chemical Components	Petroleum Ether	Acetone	Ethanol	Water
1	Steroids	+	+	+	–
2	Terpenoids	+	+	+	–
3	Cardiac glycosides	+	–	–	+
4	Alkaloids	–	+	+	+
5	Phenolic compounds	+	+	+	–
6	Tannins	–	–	+	+
7	Flavonoids	+	+	–	–
8	Saponin	–	–	–	+
9	Amino acids	+	+	+	+
10	Volatile Oils	–	+	+	–

4.4 TLC Profile of Extracts:

TLC analysis validated the presence of major bioactive groups. Each solvent yielded distinct Rf

values for different compounds, with the acetone extract displaying the most clearly resolved and intense bands.

Table 4. Rf Values of Phytochemicals in Different Solvent Extracts of *Cordyceps militaris*

S. No.	Phytochemical	Petroleum Ether (Rf)	Acetone (Rf)	Ethanol (Rf)	Water (Rf)
1	Steroids	0.44	0.56	0.75	–
2	Terpenoids	0.35	0.46	0.46	–
3	Cardiac Glycosides	0.57	–	–	0.44
4	Phenolic Compounds	0.61	0.55	0.53	–
5	Flavonoids	0.48	0.61	–	–
6	Saponins	0.66	–	–	0.67
7	Amino Acids	0.25	0.39	0.32	0.38
8	Alkaloids	–	0.74	0.74	0.39
9	Volatile Oils	–	0.70	0.97	–
10	Tannins	–	–	0.42	0.22

4.5 Antibacterial Activity of Extracts:

The acetone extract showed the strongest

antibacterial activity, particularly against *Staphylococcus epidermidis* (32 mm), *Staphylococcus aureus* (29 mm), and *Bacillus cereus* (27 mm). Petroleum ether and ethanol

extracts exhibited moderate efficacy. Water extract showed the least inhibition zones, reflecting its limited bioactive compound solubility.

Table 5. Antibacterial Activity of Different Solvent Extracts of *Cordyceps militaris* (Zone of Inhibition in mm)

S. No.	Bacterial Species	Petroleum Ether	Acetone	Ethanol	Water
1	<i>Staphylococcus aureus</i>	20	29	25	15
2	<i>Streptococcus pyogenes</i>	17	25	21	10
3	<i>E. coli</i>	12	13	19	4.4
4	<i>Pseudomonas aeruginosa</i>	9.4	23	13	8
5	<i>Staphylococcus epidermidis</i>	17	32	9	11
6	<i>Bacillus subtilis</i>	8	16	15	9.2
7	<i>Bacillus cereus</i>	10.4	27	11	–
8	<i>Yersinia enterocolitica</i>	7.1	26	23	10

4.6 HPLC-Based Cordycepin Quantification Under Environmental Variables:

Cordycepin content measured by HPLC confirmed that the optimal biosynthesis occurred at **pH 6 (6.23 mg)**, **80% humidity (5.72 mg)**, **20°C (5.62 mg)**, and **white LED light (5.21 mg)**. These results are in close agreement with visual biomass data and reflect the metabolic sensitivity of *C. militaris*.

Table 6. Effect of Environmental and Light Conditions on Cordycepin Production in *Cordyceps militaris* (HPLC Analysis)

S. No.	Factor	Condition	Cordycepin Content (mg)
1–5	Temperature	10°C–30°C	1.00–5.62
6–11	pH	4–9	2.11–6.23
12–16	Humidity	50%–90%	2.11–5.72
17–20	Light Source	Pink–White LED	2.67–5.21

4.7 HPLC-Based Cordycepin Quantification Under Nutritional Variables:

Glucose, peptone, and yeast extract emerged as the most suitable nutrient sources for maximum cordycepin yield, further confirming trends observed under UV–Vis quantification. These findings support the use of targeted carbon and nitrogen optimization in large-scale cultivation systems.

Table 7. Effect of Different Carbon and Nitrogen Sources on Cordycepin Production in *Cordyceps militaris* (HPLC Analysis)

S. No.	Factor	Source	Cordycepin Content (mg)
1–6	Carbon Source	Glucose–Starch	0.98–5.77
7–12	Nitrogen Source	Peptone–Sodium Nitrate	2.10–5.43

5.1 Environmental Factors: Temperature, pH, and Humidity:

The present study identified optimal conditions—20 °C, pH 6, and 80% relative humidity—for maximizing mycelial biomass and cordycepin production (1.21 g and 5.62–6.23 mg). These results align with earlier observations in submerged and solid-state systems indicating peak production

between 20–25 °C and pH 5–6 (Hung et al., 2009). Additionally, Jensen et al. (2024) reported increased cordycepin synthesis under controlled-liquid systems at 22 °C. Transcriptomic analyses have further demonstrated that 25 °C favors expression of key metabolic enzymes, reinforcing the importance of temperature regulation (Xia et al., 2017). Humidity at 80% was significant, echoing findings by Hung et al. (2009) who noted improved yields in surface fermentations, where moisture balance is critical for gas exchange and metabolite transport.

5.2 Nutritional Factors: Carbon, Nitrogen, and Light:

Glucose outperformed other sugars with 5.77 mg cordycepin, and organic nitrogen sources—peptone (5.43 mg) and yeast extract (5.33 mg)—proved superior. This mirrors Mao et al. (2004), who reported yields of 345 mg/L using glucose and peptone. Further, Raethong et al. (2020) optimized media via rational design, confirming that casein hydrolysate and glucose significantly improve cordycepin levels (400–600 mg/L). The efficacy of yeast extract is also supported by Zhang et al. (2022), who optimized cordycepin overproduction under pH 6. The enhanced yields are likely due to increased precursor availability for purine biosynthesis (Wang et al., 2022). The impact of white LED light aligns with recent insights into photoreceptor-mediated induction of secondary metabolites in fungi (Raethong et al., 2020).

5.3 Phytochemical Profiles:

Acetone and ethanol extracts showed strong presence of steroids, terpenoids, phenolics, flavonoids, and alkaloids, consistent with Chou et al. (2024), who reported acetone extracts of *C. militaris* are rich in diverse bioactives. Extraction methods employed reflect solvent effects noted by Asvarujanon et al. (2023), where acetone provided optimal recovery of secondary metabolites in medicinal fungi. Conversely, aqueous extracts yielded fewer compounds, echoing Barros et al. (2007) on solvent polarity effects in mushroom

phytochemistry.

5.4 Antibacterial Efficacy:

The acetone extract exhibited strong antibacterial activity—32 mm against *S. epidermidis*, 29 mm against *S. aureus*. These impressive inhibition zones surpass those reported by Rizwana et al. (2017) and Bérdy (2012), who noted 13–21 mm zones in similar fungal extracts. Heleno et al. (2015) confirmed polarity trends in ethanolic and aqueous extracts, which typically show reduced efficacy. Earlier studies on *C. militaris* by Shrestha et al. (2019) recorded 15–20 mm zones for methanolic extracts, validating the superior extraction capability of acetone. Moreover, recent work on *Ganoderma lucidum* (Asvarujanon et al., 2023) shows that acetone extracts offer enhanced antimicrobial potency.

5.5 Cordycepin Quantification by HPLC:

HPLC analysis corroborated the UV–Vis-based findings and established that environmental and nutritional optimization enhances cordycepin yield. These findings parallel those of Zhou et al. (2016), where submerged culture with yeast extract and tryptone at pH 6 produced 7.35 g/L cordycepin. In addition, Huang et al. (2023) enhanced production to 6.54 g/L by utilizing xylose-utilizing strains, indicating the potential of custom sugar utilization to further improve yields. Other studies also confirmed that elicitors like plant oils and complex media can raise cordycepin yields beyond 5 g/L (Xia et al., 2017).

5.6 Implications and Future Directions:

The present study successfully establishes optimal culture parameters—namely 20–25 °C temperature, pH 6, and 80% relative humidity—for enhanced mycelial growth and cordycepin production in *Cordyceps militaris*. These findings are consistent with earlier reports and hold significant potential for scale-up applications in industrial bioprocessing. Nutritional optimization further revealed glucose as the most effective carbon source, while peptone and yeast extract emerged as the superior nitrogen sources, confirming previous studies on the metabolic benefits of organic nitrogen compounds for purine biosynthesis (Mao et al., 2004; Zhang et al., 2022). The use of white LED lighting demonstrated an additional benefit in stimulating secondary metabolite synthesis through photoreceptor-mediated pathways, thus offering a controllable environmental factor to enhance productivity (Raethong et al., 2020). Among extraction strategies, acetone proved to be the most effective solvent, yielding phytochemically rich and highly bioactive extracts, which is consistent with its established efficiency in fungal metabolite extraction (Chou et al., 2024).

To further enhance cordycepin yield and standardize production protocols, several advanced strategies are recommended. Statistical tools such as Response Surface Methodology (RSM) and Box–Behnken Design (BBD) have shown success in media optimization and could be utilized to refine nutritional balance and environmental parameters (Wang et al., 2024). Elicitors such as plant oils, casein hydrolysate, and amino acid supplements have demonstrated efficacy in promoting secondary metabolism and should be tested in combination with optimized media (Raethong et al., 2020; Zhang et al., 2022). Additionally, genetic approaches including CRISPR/Cas-mediated editing and targeted metabolic engineering offer promising avenues for constructing high-yield strains (Duan et al., 2023; Wang et al., 2022). Process scale-up using bioreactor systems is essential for industrial application, where parameter control and reproducibility are critical. Finally, integration of omics tools such as transcriptomics and metabolomics can uncover regulatory pathways involved in cordycepin biosynthesis and enable precision-driven enhancement of production, as highlighted in recent studies by Xia et al. (2017). Collectively, these strategies can transform *Cordyceps militaris* into a commercially viable platform for large-scale production of cordycepin and related bioactives.

6. CONCLUSION:

The present study systematically investigated the influence of various **environmental, nutritional, and physical parameters** on the growth and **cordycepin production** of *Cordyceps militaris* under controlled cultivation conditions. A combination of empirical optimization, phytochemical screening, antibacterial evaluation, and HPLC-based quantification was employed to establish the ideal culture settings.

Among environmental variables, **temperature (20°C), pH (6.0), and humidity (80%)** were found to significantly enhance both biomass and cordycepin yield. These results are in concordance with previous studies that emphasize the thermosensitivity and pH-dependency of fungal secondary metabolite pathways. Nutritional parameters further played a critical role—**glucose** emerged as the most effective carbon source, while **peptone** and **yeast extract** outperformed inorganic nitrogen sources, reinforcing the metabolic need for rich organic nutrients in cordycepin biosynthesis. The study also confirmed that **white LED light** positively influenced cordycepin content, likely by activating light-responsive biosynthetic genes. This photostimulatory effect opens new avenues for photobioreactor-based cultivation. Solvent

extraction and TLC revealed that **acetone extracts** had the richest phytochemical content, including steroids, terpenoids, alkaloids, and phenolic compounds, which correlated well with their superior **antibacterial activity**, particularly against *Staphylococcus epidermidis*, *Bacillus cereus*, and *Staphylococcus aureus*.

Quantitative analysis through **HPLC** validated that optimal environmental and nutritional conditions could significantly amplify cordycepin levels, with concentrations peaking at 6.23 mg under ideal settings. These findings underscore the strong relationship between culture optimization and bioactive compound productivity in *C. militaris*.

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