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Optimization of media components for the improved Production of Cordycepin with *Cordyceps militaris* by Liquid State fermentation

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Abstract
Background: *A member of Ascomycota, the highly valued medicinal fungus, Cordyceps militaris has attracted considerable attention owing to its production of numerous bioactive compounds, including polysaccharides, carotenoids, and nucleosides, such as cordycepin, all of which have significant nutritional and therapeutic potential. Cordyceps militaris produces the nucleoside analog cordycepin (3'-deoxyadenosine), which has a variety of effects, including immunological control, anti-cancer, anti-fungal, anti-leukemia, and anti-hyperlipidemia qualities. Researchers worldwide have focused on in vitro culture because of its unique perspective and the necessity of its biomolecules. The current study focused on enhancing the fermentation conditions for C. militaries to boost cordycepin production and its applications.*
Methods: *To study the effect of media components on cordycepin production, various carbon, nitrogen sources, growth factors such as amino acids and vitamins, inoculum size, and age were used in the fermentation medium.*
Results: *The ideal media components were 15g/L glucose as the carbon source, 15g/L yeast extract as the nitrogen source, 1g/L tri-ammonium citrate as the inorganic salt, 12g/L Histidine and Vitamin B12 as growth factors. Under liquid fermentation at 25°C and pH 5, Cordyceps militaris produced a 3.5 fold increase in cordycepin production per liter.*
Conclusion: *This study was conducted to establish the key factors that improve cordycepin production by C. militaris. The optimal medium contained glucose (1.5 %), yeast extract (1.5 %), and 1% tri-ammonium citrate as inorganic salts, histidine, and vitamin B12 as inducers. A 10% three-day-old inoculum was found to be optimum at 25°C, pH 5 for 35 days. Using these culture conditions, a maximum production of cordycepin obtained was 7.95 g/L which is 3.5 fold increase than that of the control. This method provides an effective way to increase cordycepin production in liquid-state fermentation.*
Key words: *Cordyceps militaris, cordycepin, Liquid State Fermentation (LSF)*
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I. Introduction:

Cordyceps spp. mushrooms, commonly known as caterpillar fungus, are classified into the Ascomycota group and have a long tradition as a natural agent in Asia. *Cordyceps militaris (C. militaris)* is a well-known entomopathogenic and medicinal fungus that has long been used in traditional medicine in East Asia, has garnered substantial scientific interest owing to its production of biologically active metabolites, particularly cordycepin. (Showkat *et al*., 2024). It is known for its umami flavor, aroma, nutritional value, and diverse medicinal properties, including antioxidant, anti-inflammatory, immunomodulatory, and antitumor activities (Song *et al.,* 2025). These functional properties are due to its rich content of bioactive substances, such as cordycepin, polysaccharides, and sterols.

Cordycepin, an adenosine analog (3′-deoxyadenosine), from *C. militaris*, is one of its biologically active components widely used as a food-based tonic and herbal remedy in China, Japan, and other Asian countries (Jiapeng *et al.,* 2013). Cordycepin exhibits various physiological and pharmacological activities, including antitumor, antileukemic, antimetastatic, antibacterial, antiviral, antitrypanosomiasis, antirestenosis, immunomodulatory, and anti-inflammatory activities (Paterson & R.R.M 2008; Ng and Wang 2005). These activities make cordycepin a promising molecule for use in pharmaceuticals, nutraceuticals, and functional foods. However, low yields and high production costs remain major bottlenecks for commercialization and industrial applications (Jeennor *et al.,* 2023; Li *et al.,* 2024). Achieving economically viable cordycepin production requires not only the selection of high-yielding strains but also the optimization of the fermentation

process, including culture medium composition, physical parameters such as temperature, pH, and agitation, fermentation mode such as static, submerged, solid-state, and semi-solid, inoculum size, and seed age (Kang *et al.,* 2014; Wen *et al.,* 2016; Showkat *et al.,* 2024). Even after many scientific studies on fermentation and optimization of cordycepin there are few challenges like while optimizing one parameter (e.g. temperature) may negatively affect another (e.g. biomass vs secondary metabolite yields) (Adnan *et al.,* 2017). The interactions among the parameters are often nonlinear. Laboratory-scale optimization does not always translate well to pilot or industrial scales. Parameters such as aeration, mixing, oxygen transfer, heat removal, and substrate heterogeneity (in solid state) can behave differently at large volumes (Mao and Zhong 2004; Park H.J. 2025). Despite extensive research, no clear consensus has emerged regarding which fermentation mode—liquid, static, submerged, semi-solid, or solid-state—offers the most cost-effective and scalable production of cordycepin. Given these considerations, there is a need for further systematic optimization of fermentation conditions for *C. militaris* with the goal of maximizing cordycepin yield under cost-effective, scalable, and reproducible conditions. Specific objectives of the present work include evaluating and optimizing nutritional (carbon, nitrogen, and mineral sources) as well as inoculum size and age parameters.

II. MATERIALS AND METHODOLOGY

Materials:

Standard cordycepin was purchased from Sigma-Aldrich, and potassium dihydrogen phosphate (KH2PO4), dipotassium hydrogen phosphate (K2HPO4), tri-ammonium citrate ((NH4)3C6H5O7), ferrous sulfate (FeSO4), calcium chloride (CaCl2), zinc sulfate (ZnSO4), and magnesium sulfate (MgSO4), glucose, fructose, galactose, sucrose, maltose,

Lactose, starch, cellulose, and cellobiose, beef extract, casein, peptone, and yeast extract, sodium nitrate ($NaNO_3$), ammonium nitrate (NH_4NO_3), ammonium sulfate (NH_4SO_4), Histidine, Leucine, Lysine, Vitamin C, Biotin, B6, B12, and methanol were of high grade quality.

Microorganism:

A pure culture of *Cordyceps militaris* was purchased from the ICAR-Directorate of Mushroom Research, Chambaghat, Solan, (H.P) (Kumar.S *et al*., 2023). The obtained pure culture was stored at 4°C in laboratory and maintained on Potato Dextrose Agar (PDA) slants or plates at 25 °C for 7 days.

Preparation of Inoculum: The basal medium was prepared by adding sucrose (20 g/L), peptone (20 g/L), KH_2PO_4 (1 g/L), and $MgSO_4.7H_2O$ (0.5 g/L) and sterilized.

Inoculation and Fermentation:

50mL of fresh basal medium was added to the active PDA slant of the stock culture. The spores were scraped using a sterilized inoculation loop, and the medium was introduced into a sterilized 250mL flask and the culture was incubated at 25°C on a rotatory shaker at 150 rpm for 5 days to develop the inoculum.

Effect of Carbon sources

Effects of various carbon compounds namely, glucose, fructose, galactose, sucrose, maltose,

Lactose, starch, cellulose, and cellobiose on cordycepin production by *C. militaris* were studied. The broth was distributed into different flasks, and various carbon sources (1.5 %) were added to each flask prior to inoculation of the strain. Medium lacking any carbon source served as the control.

Effect of Nitrogen sources:

The effects of complex organic (beef extract, casein, peptone, and yeast extract) and inorganic nitrogen sources (sodium nitrate, ammonium nitrate, and ammonium sulfate) on cordycepin production were studied. Each nitrogen source was added individually to the basal medium at a concentration of 1.5 %. Nitrogen free medium is used as control.

Effect of growth factors

To identify the effect of growth factors for the production of cordycepin, a few amino acids (Histidine, Leucine, Lysine) and vitamins, such as Vitamin C, Biotin, B6, B12, were used at a concentration of 1.2 %.

Effect of inorganic salts:

To study the effect of inorganic salts on cordycepin production, various salts, such as potassium dihydrogen phosphate (KH2PO4), dipotassium hydrogen phosphate (K2HPO4), tri-ammonium citrate ((NH4)3C6H5O7), ferrous sulfate (FeSO4), calcium chloride (CaCl2), zinc sulfate (ZnSO4), and magnesium sulfate (MgSO4), were used at a concentration of 1%.

Effect of inoculum age:
To study the effect of inoculum age on cordycepin production, the inoculum developed for different time intervals (1-4 days) was used to inoculate the fermentation medium.
Effect of inoculum size:
Different volumes of inoculum were added to the fresh fermentation medium containing glucose 15 g/L, yeast extract 15 g/L, KH_2PO_4 1.0 g/L, $MgSO_4.7H_2O$ 1.0 g/L, tri ammonium citrate 1.0 g/L, Histidine 12 g/L, and Vitamin B12 12 g/L.
Fermentation conditions:
All flasks were incubated at 25°C under static conditions for 35 days with exposure to light and dark conditions for 15 days each. All experiments were performed in triplicate.
Recovery:
The fermented broth was filtered, and the mycelium was separated, sufficiently washed with distilled water, and dried at 110°C in an oven until a constant dry weight was obtained. The resulting supernatant was used to measure the cordycepin content.
Purification of Cordycepin:
The fermentation broth was boiled for 30 min and centrifuged at 8000Xg for 30 min at 4°C. The supernatant was mixed with four volumes of chilled ethanol and left to stand at 4℃ overnight. The resultant precipitate was removed by centrifugation at 8000Xg for 30 min. The supernatant was then mixed with activated charcoal powder and kept at 80℃. After 4hrs, the sample was centrifuged at 7200Xg for 10 min, the supernatant was dried in a rotary evaporator, and the wet filter cake was recrystalized from 20mL of hot ethanol three times. Crude cordycepin was obtained (Tang *et al.,* 2013).
Analytical methods:
The cordycepin content was determined using a UV-Vis spectrophotometer at 260 nm (Krishna *et al.,* 2024). All samples were run in triplicate.
Statistical analysis:
The effects of optimized nutritional sources such as carbon, nitrogen, inorganic salts, and growth factors were subjected to fermentation with different volumes of inoculums and age were analyzed by Anova single factor test.

III. Results :

The pure culture of *Cordyceps militaris* obtained on PDA medium was inoculated into liquid basal medium. The inoculum developed in the liquid basal medium was used for the optimization studies under liquid state fermentation.

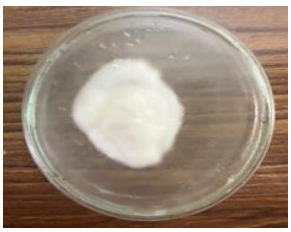

Fig 1: Culture on PDA

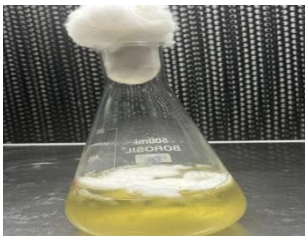

Fig 2: Inoculum in basal medium

Fig 3: Fermentation in LSF

Effect of carbon source:
The yield of cordycepin (mg/L) is significantly influenced by the medium containing different kinds of carbon source (1.5%). Cordycepin biosynthesis is greatly enhanced by easily accessible hexose and disaccharide carbon sources, as seen by the highest yield of glucose (650 mg/L) and sucrose (620 mg/L) among the studied sugars. sugars like starch (500 mg/L) and maltose (450 mg/L) yield substantial amounts. While lactose, galactose, and fructose continue to be relatively poor carbon sources (350-380 mg/L), These findings are consistent with earlier research demonstrating that the best carbon source for Cordyceps militaris to produce cordycepin in liquid static culture is glucose (Mao *et al*., 2005; Sirithep *et al*., 2020).

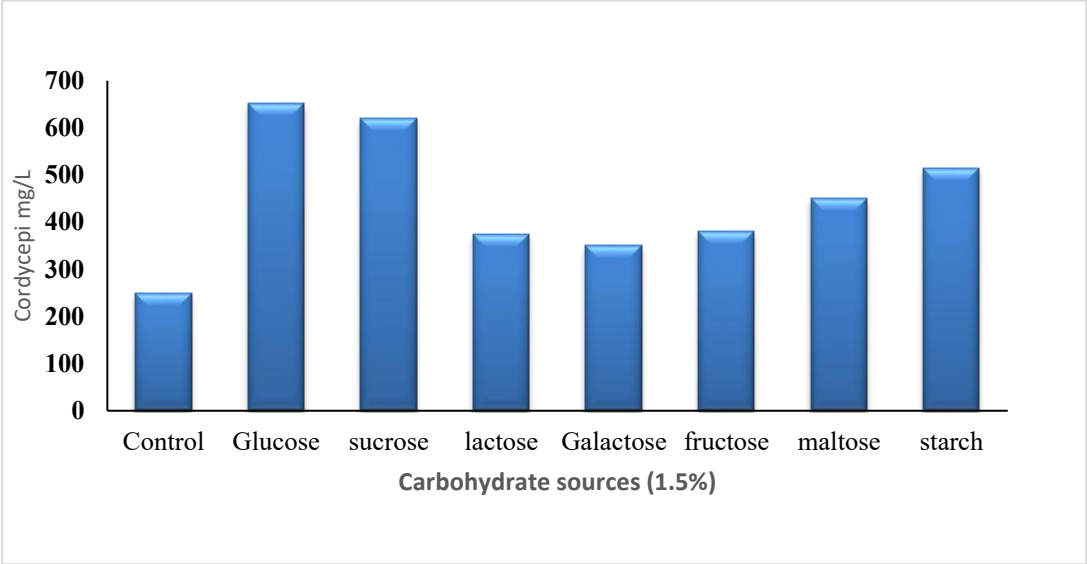

Fig 4: Effect of carbon source on cordycepin production

Effect of nitrogen source:
The findings show that the type of nitrogen source (1.5%) has a significant impact on the production of cordycepin in liquid cultures of *Cordyceps militaris*. While inorganic sources ($NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$) support only moderate production (380-420 mg/L), organic sources such as yeast extract, 650 mg/L, and peptone, 620 mg/L, considerably increase cordycepin yield.

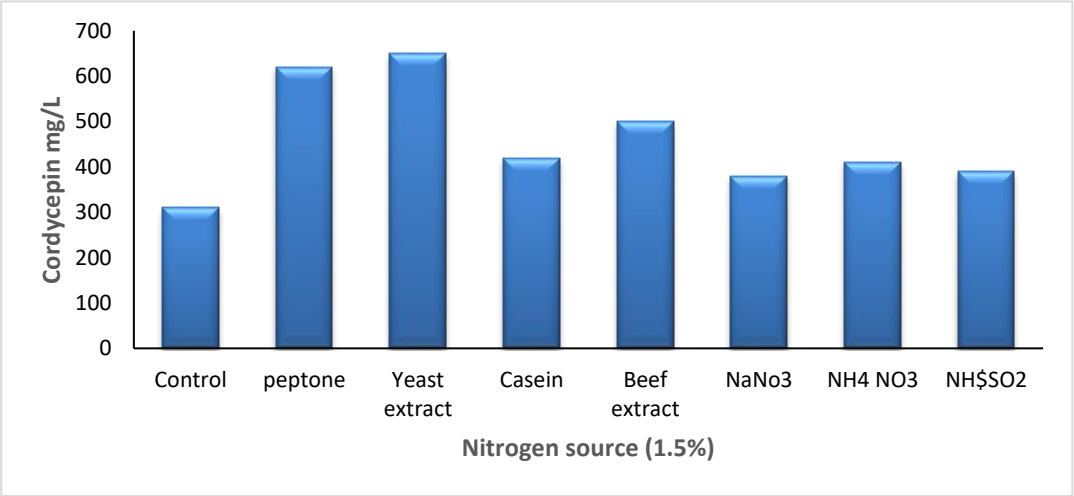

Fig 5: Effect of nitrogen source on cordycepin production

Effect of Inorganic salts:
The effects of many inorganic salts at 1% on cordycepin production were investigated, including KH_2PO4, K_2HPO_4, triammonium citrate, ferrous sulphate ($FeSO_4$), calcium chloride ($CaCl_2$), magnesium sulphate ($MgSO_4$), and zinc sulphate ($ZnSO_4$). According to the results, $ZnSO_4$ produced the least amount of cordycepin (260 mg/L), while triammonium citrate produced the most (over 300 mg/L). Triammonium citrate > $MgSO_4$ > $FeSO_4$ > $CaCl_2$ > KH_2PO_4 > K_2HPO_4 > $ZnSO_4$ was the order of effectiveness on cordycepin production. According to these results, the best inorganic salt for increasing cordycepin synthesis is triammonium citrate.

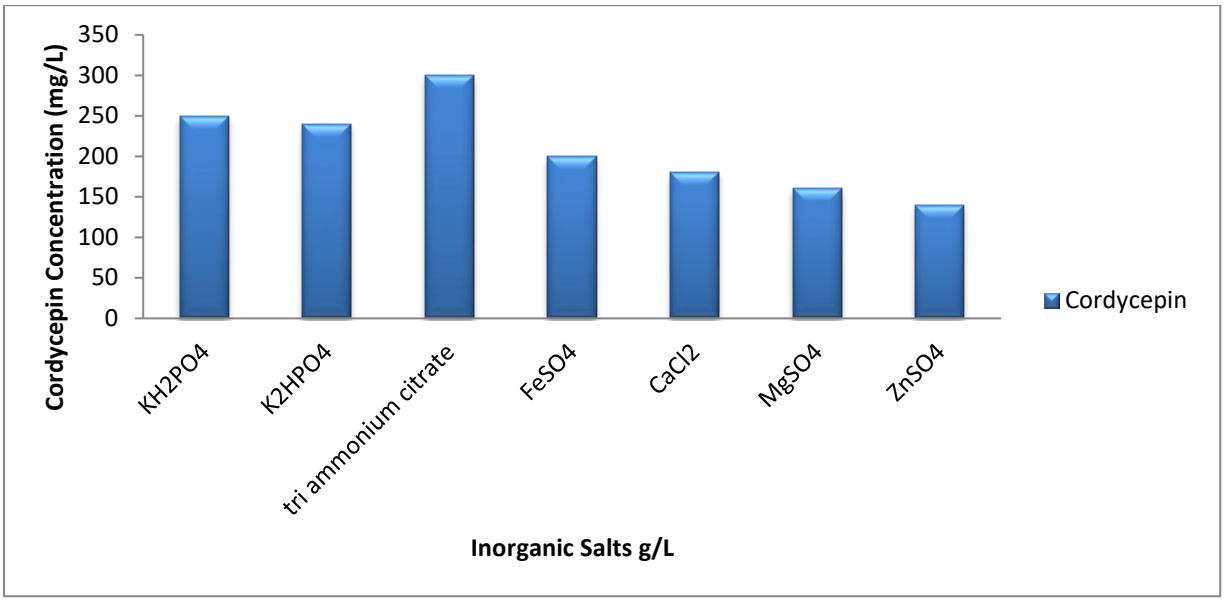

Fig 6: Effect of inorganic salts on cordycepin production

Effect of Growth factors

The impact of several growth factors (1.2 %) on cordycepin production was assessed, and the findings showed that the type of supplement supplied significantly affected the cordycepin output. Nucleoside biosynthesis and associated metabolic pathways are strongly influenced by vitamin B12, which promoted the highest cordycepin production of the studied substances, reaching 450 mg/L. Additionally, Histidine significantly increased the accumulation of cordycepin (400 mg/L), indicating that this amino acid may be important in promoting nitrogen availability and the enzymatic processes involved in cordycepin synthesis.

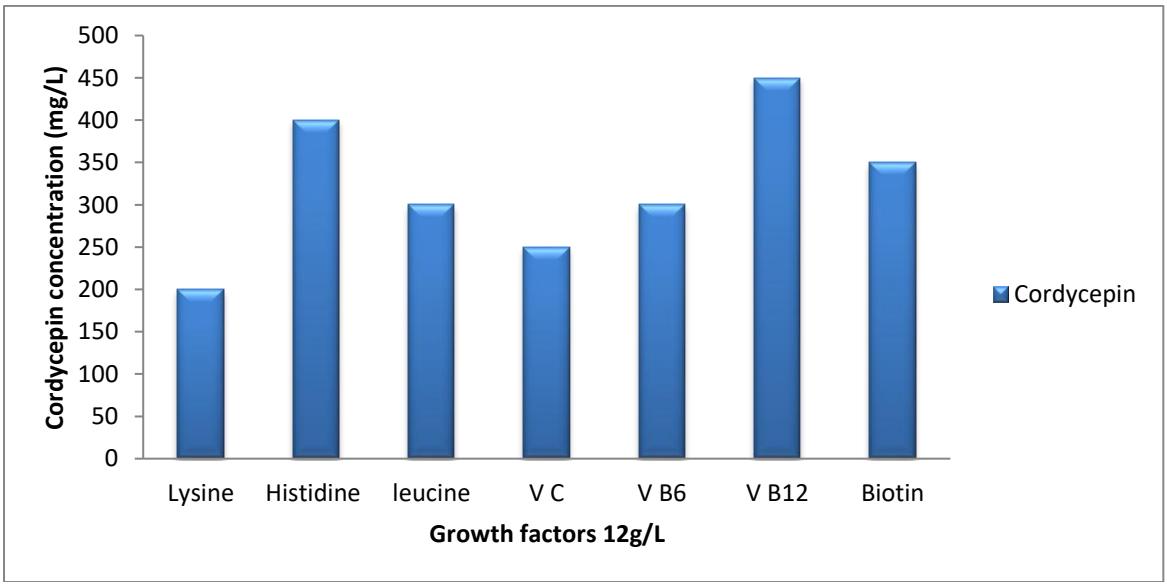

Fig 7: Effect of growth factors on cordycepin production

Effect of inoculum size and age on optimized fermentation media:

After optimizing the nutritional factors of fermentation media, components are glucose 15/L, yeast extract 15g/L, tri ammonium citrate 1g/L, Histadine 12g/L and Vitamin B 12 12g/L along with basal medium. The combined effects of these nutritional factors were studied by varying inoculum size and age, the results are represented below in Figure: 8 and Fig:9 respectively. In this study, we observed that inoculum size of 10% and 3-day old inoculum yields high production of cordycepin. Final fermentation with optimized inoculum size and age results in 3.5 fold increase i.e., 7.95g/L than that of control with 4.45g/L.

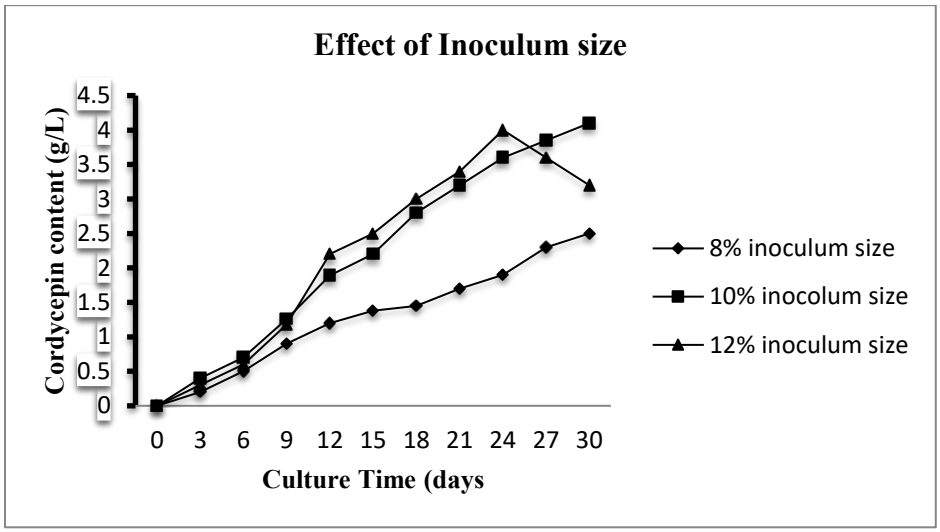

Fig 8 : Effect of inoculum size on cordycepin production

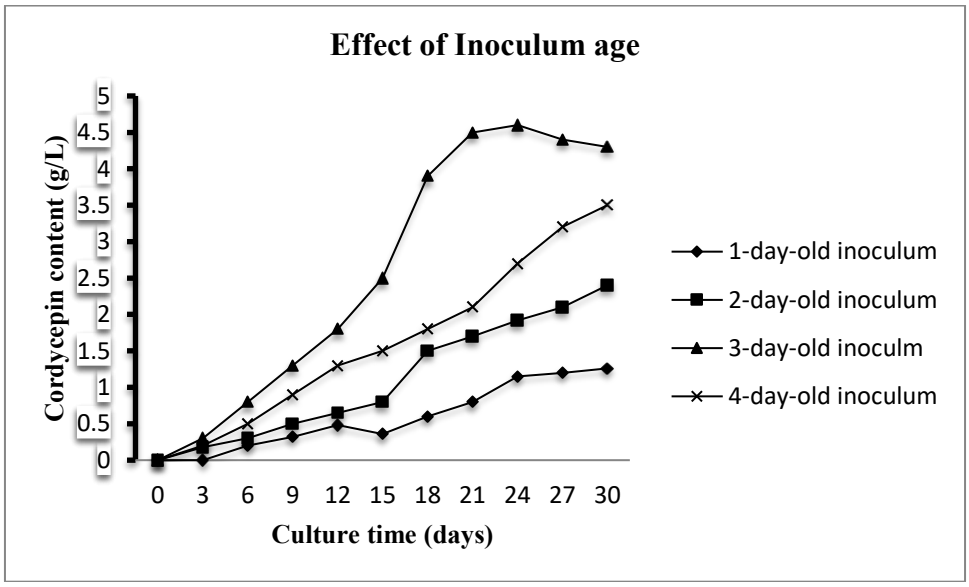

Fig 9: Effect of inoculum age on cordycepin production

Statistical analysis:

Anova: Single factor analysis for inoculum size

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
0.08	11	14.03	1.275455	0.669227
0.1	11	29.85	2.713636	2.553165
0.12	11	21.2	1.927273	2.121682

Anova

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	11.40921	2	5.704603	3.20239	0.054884	3.31583
Within Groups	53.44075	30	1.781358			
Total	64.84995	32				

A one-way ANOVA was conducted to evaluate the effect of different inoculum sizes (0.08, 0.10, and 0.12) on the measured response variable. The summary statistics show noticeable variation among the group means: 0.08 produced an average value of 1.27, 0.10 resulted in the highest mean of 2.71, while 0.12 yielded a mean of 1.93. Variance values also differed, with the 0.10 inoculum size showing the highest variability. The F-value is 3.20239**,** while the critical F-value at $\alpha = 0.05$ is 3.31583**.** Since F < Fcrit**,** the null hypothesis cannot be rejected at the 5% significance level. Similarly, the obtained p-value is 0.054884**,** which is slightly above the 0.05 threshold. This confirms that there is no statistically significant difference among the three inoculum sizes at the 95% confidence level.

Anova: Single factor analysis for inoculum age

Summary

Groups	*Count*	*Sum*	*Average*	*Variance*
one day	11	6.5	0.590909	0.212449
two day	11	12.05	1.095455	0.723107
three day	11	28.4	2.581818	3.305636
four day	11	17.7	1.609091	1.398909

Anova

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	23.85426	3	7.95142	5.639203	0.00255	2.838745
Within Groups	56.40102	40	1.410025			
Total	80.25528	43				

The ANOVA results for the single-factor analysis on inoculum age indicate a statistically significant difference between the means of the groups (one day, two day, three day, four day). This is evident from the P-value of 0.00255, which is less than the typical significance level of 0.05, and the F-value of 5.639203 being greater than the F critical value of 2.838745. Looking at the group means, the averages are 0.590909 for one day, 1.095455 for two days, 2.581818 for three days, and 1.609091 for four days. The three-day group has the highest average. Overall, the results suggest that inoculum age has a significant effect on the outcome being measured.

IV. Discussion:

The present study demonstrated that the systematic optimization of nutritional components in liquid state fermentation significantly enhanced cordycepin production by *Cordyceps militaris.* The findings underscore that strong dependence of cordycepin biosynthesis on carbon and nitrogen metabolism, mineral balance and growth promoting factors, all of which modulate fungal physiology and secondary metabolite pathways. Key optimization strategies for liquid fermentation include screening high-cordycepin–producing strains, applying ion-beam irradiation, adding functional agents to the medium, optimizing medium composition, employing two-stage shaking–static cultivation, and using LED irradiation (Kang *et al.,* 2017). Liquid culture is the other option for the cultivation of *C.militaris*. Liu, SR and Zhang 2019 considered it as a better procedure for application in industry due to its shorter cultivation times, smaller space requirements, and higher productivities. Cui, J. D. 2014 cultured *Cordyceps militaris* in liquid as mycelia, without forming fruiting bodies, and total cultivation time decreased from 60 to 15 days. Our results indicated that liquid static fermentation for 35 days, markedly influenced cordycepin production. The present findings align with earlier reports showing that moderate glucose concentrations favor cordycepin production, while higher concentrations prioritize biomass formation rather than metabolite synthesis (Mao & Zhong, 2004; Liang *et al.,* 2009; xiao *et al*., 2019 Das *et al*., 2010). Experimental results Anantayanon *et al*., 2025 revealed that the optimal culture components involved 30.0 g/L of glucose, 9.8 g/L of yeast extract, and 1.5 g/L of adenine, obtained maximum cordycepin concentration (1724.53 ± 18.30 mg/L) with a short fermentation time of 2 days. Carbon and nitrogen sources are key determinants of liquid fermentation performance, as they directly affect cell growth and metabolite synthesis. Using an optimized combination of 42.0 g/L glucose and 15.8 g/L peptone, cordycepin production reached 0.35 g/L, representing a 40% increase over the control. (Mao *et al.,* 2005; Ha, S.Y.*et al.,* 2020). Chang, Y *et al.,* 2024 observed that the combination of peptone and Corn Steep Liquor Hydrolysate (CSLH) served as the most advantageous nitrogen source for cordycepin production, leading to a maximum production of 277.29 ± 18.60 mg/L. In our studies the yeast extract effectiveness may enhances cordycepin

production when combined with either Corn Steep Liquor, CSLH or sugarcane baggase. Nitrogen is a major determinant of fungal secondary metabolism, and its form greatly impacts cordycepin production. Organic nitrogen sources such as yeast extract and peptone enhanced cordycepin accumulation more effectively than inorganic nitrogen. This may be due to the presence of vitamins, amino acids, and complex nutrients that stimulate enzymatic activity and improve precursor availability. Previous studies similarly reported that organic nitrogen promotes cordycepin synthesis, while excessive nitrogen represses secondary metabolism (Hsu *et al*., 2002; Liang *et al*., 2009). These findings indicated that the selection of nitrogen source plays a significant role in cordycepin synthesis. The present study carried out with different carbon and nitrogen sources, yields high amount of cordycepin 650 mg/L with glucose and yeast extract similarly indicates the influence of carbon and nitrogen sources on cordycepin synthesis. Gang *et al.,* 2016, in comparison to various inorganic salts in liquid culture maintained at 23°C, stated that K_2HPO_4 and $MgSO_4$.$7H_2O$ were beneficial for *C. militaris* mycelial growth and biomass accumulation. According to our study, the above mentioned salts are considered as basal media components along with these two salts tri ammonium citrate boosted the production of codycepin. Yu, W., *et al.,* 2024 identified that alanine is a pivotal factor that improve Cordycepin production in the culture medium, which serves a critical role as a precursor in the biosynthesis of Cordycepin. Supplementing the culture media with 8 g/L L-alanine has resulted in a remarkable two fold increase in Cordycepin concentration. Wen *et al*.,2016 studied the effect of aminoacid for cordycepin production by solid state fermentation by adding different amino acids at a concentration of 4%, among various kinds, lysine, 7.18+1.12 mg/g histidine 6.95+1.01mg/g,and glycine7.08 + 0.90mg/g greatly improved cordycepin production. Masuda *et al.,* (2007) reported that L-glycine, L-alanine, L-aspartic acid, L-glutamine, and adenine enhanced cordycepin production, with the optimal additive combination—1 g/L adenine and 16 g/L glycine—yielding 2.50 g/L cordycepin, a 4.1-fold increase compared with the basal medium, In our study, we supplemented the media with 12 g/L – Histidine, interestingly we found that, it uplifted cordycepin accumulation (400mg/L) and in final optimized fermentation a 3.5 fold increase is observed compared with control, suggesting that this amino acid may be important in promoting nitrogen availability and the enzymatic processes involved in cordycepin synthesis, and selection of agriculture waste having more amount of Histidine, alanine, lysine, and glycine may enhance the production widely. Supplementation with growth factors including vitamins and amino acids, further boosted cordycepin output vitamins particularly B complex groups acts as essential cofactors in nucleic acid metabolism and energy pathways, growth enhancers have been previously reported to stimulate cordycepin formation by improving precursor flow and enhancing physiological activity (Ruan Yuan et al., 2014; Xiao *et al*.,2019; Park *et al.,* 2020), This prompted us to assess the impact of vitamins on cordycepin production, we analysed it with four different types of vitamins namely vitamin B12, B6, biotin, and vitamin C, among them vitamin B12 showed significant impact on nucleoside biosynthesis and associated metabolic pathways, as evidenced by the highest cordycepin production, which reached 450 mg/L. In this study the effective stimulation of cordycepin production following supplementation highlights the importance of micro nutrient support in optimizing secondary metabolite synthesis in *C. militaris*. S.k Das *et al*., 2010 explored the effect of inoculants on the production of cordycepin in surface liquid culture, several inoculants single (control), double and triple were used. Their analysis showed that the cordycepin production in control was 5.05g/L, double 6.11 g/L and triple 5.61g/L respectively. Tuli *et al*., 2014 observed that cordycepin production (304mg/L) was maximum when 8% (v/v) of 72 hours old inoculums was added to the production medium. In our work, it was found that fermentation with optimum inoculum size 10% and 3-day old culture leads to a 3.5-fold increase in cordycepin production (7.95g/L). Moreover our studies showed that there is a less significant difference in production of cordycepin by varying inoculum sizes. The overall improvement in cordycepin yield demonstrates the practicality and scalability of optimizing nutritional components in LSF. Compared with solid-state fermentation, LSF offers better control over pH, oxygen distribution, and nutrient homogeneity, leading to more consistent and enhanced cordycepin production (Zhang *et al.,* 2017). The integrated optimization strategy employed in this study—addressing carbon and nitrogen sources, mineral salts, and growth factors—resulted not only in higher productivity but also in improved substrate conversion efficiency, affirming the robustness of medium engineering for industrial applications. Moreover, cordycepin degradation during fermentation remains an industrial challenge. Combining nutritional optimization with metabolic or genetic engineering may further enhance cordycepin productivity.

V. CONCLUSION:

Process optimization revealed that *C. militaris* exhibits maximal cordycepin biosynthesis when cultivated under a carbon-rich, nitrogen-balanced regime comprising glucose, yeast extract, and tri-ammonium citrate, supplemented with metabolic enhancers histidine and vitamin B12 evaluation indicated that inoculum physiological state—specifically a 72-hours maturation period—significantly modulates secondary metabolite synthesis, whereas inoculum volumetric load showed statistically relevant effect. Integration of all optimized parameters enhanced cordycepin titers to 7.95 g/L, demonstrating a 3.5-fold elevation. These findings establish

a scalable, high-efficiency fermentation platform and provide a strong foundation for future reactor-level optimization and systems-biology-driven strain engineering.

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