Enhanced production of cordycepin by medium engineering in *Cordyceps militaris*

Thesis submitted in partial fulfillment of the requirement for the degree of

Master of Science

In

Microbiology

By

Pallavi Sharma (235112003)

Under the supervision

of

Dr. Ashok Kumar Nadda

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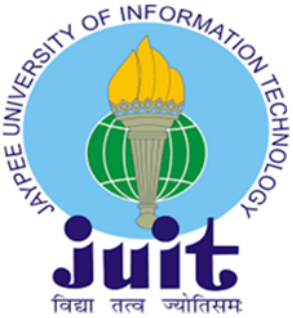

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DECLARATION

I hereby declare that the work presented in this report entitled "Enhanced production of Cordycepin by medium engineering in *Cordyceps militaris*" in partial fulfillment of the requirements for the award of the degree of Master of Science in Microbiology submitted in the Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat is an authentic record of my own work carried out over a period from August 2024 to May 2025 under the supervision of Dr. Ashok Kumar Nadda, Assistant Professor, Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Solan, Himachal Pradesh.

The matter embodied in the report has not been submitted for the award of any other degree or diploma.

PALLAVI SHARMA (235112003)

This is to certify that the above statement made by the candidate is true to the best of my knowledge.

Dr. Ashok Kumar Nadda

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Dated:

CERTIFICATE

This is to certify that the work reported in the M.Sc. Microbiology thesis entitled "Enhanced production of cordycepin by medium engineering in *Cordyceps militaris*", submitted by Ms. Pallavi Sharma (235112003) at Jaypee University of Information Technology, Waknaghat, India, is a Bonafide record of her original work carried out from August 2024 to May 2025 under my supervision. This work has not been submitted elsewhere for any other degree or diploma

Dr. Ashok Kumar Nadda

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Date:

ACKNOWLEDGEMENT

I would like to extend my heartfelt gratitude to **Dr. Sudhir Kumar**, Professor and Dean of Research and Development, JUIT, for his valuable guidance and unwavering support throughout this project. His encouragement and cooperation at every stage were truly motivating and deeply appreciated

I am equally grateful to **Dr. Jata Shankar**, Head of the Department of Biotechnology and Bioinformatics, JUIT, for his insightful suggestions, continuous support, and encouragement from the initiation to the successful completion of this work.

My sincere thanks go to my project supervisor, **Dr. Ashok Kumar Nadda**, Assistant Professor, Department of Biotechnology and Bioinformatics, JUIT. His dedicated mentorship, continuous guidance, and valuable feedback were instrumental in shaping this project. I am deeply thankful for his consistent support and encouragement throughout the journey.

I also wish to acknowledge the support of Ph.D. scholars for their constant motivation, assistance, and helpful suggestions, which greatly contributed to the completion of my work.

I am thankful to all the technical and laboratory staff of the Department of Biotechnology and Bioinformatics for their kind assistance and cooperation, which facilitated the smooth conduct of my research.

Finally, I would like to express my sincere appreciation to my parents and my friends for their continuous support, encouragement, and companionship throughout this project.

Thank you one and all.

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ABSTRACT

Cordycepin (3'-deoxyadenosine) is a bioactive compound with diversity of pharmacological activities, including anti-inflammatory, anticancer, and immunomodulatory effects. *Cordyceps militaris*, a well-known medicinal mushroom, is an abundant natural source of cordycepin. However, the yield of cordycepin from *C. militaris* in typical cultivation methods is often suboptimal, prompting interest in medium engineering strategies to enhance its production. This study investigates various approaches to optimizing the growth conditions and metabolic pathways of *C. militaris* using medium engineering to increase cordycepin production. The effects of different carbon sources, nitrogen sources, trace elements, and pH levels on fungal growth and cordycepin synthesis were thoroughly studied. The findings reveal that optimized medium conditions, such as balance of glucose and peptone, enhanced nitrogen levels, and the incorporation of specific trace elements, significantly increased cordycepin production. This research provides valuable insights into medium engineering for the large-scale production of cordycepin and can serve as a foundation for future biotechnological applications in the pharmaceutical industry.

Key words:*C. militaris*, Cordycepin, Optimization,Medium Engineering.

CHAPTER – 1

INTRODUCTION

Medicinal mushrooms have gained significant attention in recent years for their bioactive compounds and therapeutic properties. Among these, Cordyceps is a genus of fungi with over 400 known parasitical fungal species. Cordyceps are well grown in humid and warm climate, particularly in Tibet and China. Cordyceps has been recognized as more beneficial medicinal treatment in Asian countries. Two species of mushroom have received worldwide attention such as *Cordyceps sinensis* and *Cordyceps militaris*. The *C. sinensis* is difficult to cultivate. While, *C. militaris* might grow fruiting bodies on cereal grains like rice.It is widely recognized by its remarkable pharmacological activities, including anti-tumor, immunomodulatory, antioxidant, and anti-inflammatory effects **[1].** The primary bioactive compound attributed to these effects is Cordycepin. Cordycepin (3'-deoxyadenosine) is one of the most significant bioactive metabolites produced by *C. militaris*, a parasitic fungus, because of its broad range. The *C. militaris is* expensive due to certain fruiting bodies which needs time to produce and results in a small size. Therefore, the *C. militaris* provides less productions for drug and food supplements. In order to obtain high production of *C. militaris* and fulfill the requirement of the customers in the world markets the researchers used different types of cereals in Solid Substrate Fermentation such as rice, wheat, and oat to cultivated the variety of species of *C. militaris***[2].**

Traditionally used in East Asian medicine, *C. militaris* has gained commercial and scientific interest due to is ease of artificial cultivation. One of the major challenges in *C. militaris*cultivationis the optimization of growth conditions to enhance the yield of cordycepin. Several studies have shown that environmental parameters and nutritional composition of the medium significantly influence both fungal biomass and metabolite synthesis. Therefore, medium engineering the strategic modification of culture substrates and nutrient profiles has emerged as a promising approach to improve both mycelial growth and bioactive compound production **[3].**

Rationale of the study

Although synthetic and chemically defined media have been used for fungal cultivation, the use of cost-effective and sustainable agricultural substrates provide dual benefits : reducing production cost and utilizing agro-waste efficiently. Substrates such as rice, soybean,

corncob, mungbean, barley have been explored to support fungal development, but their comparative efficacy, particularly in enhancing cordycepin yield, remain inadequately studied under standardized conditions**[4].**To cultivated the *C. militaris* on cereal grains, the aseptic techniques were required. For solid media cultivation, four cereals were tested including brown rice (control), brown rice with different substrates**.** The solid media cultivation prepared by two steps involved Liquid Media Portion (LMP), where applied substrate residue as main protein sources for supporting the growth of fruiting bodies, and Solid Media Portion (SMP) used rice brown as main nutrients**[4].**

This special project aimed at enhancing *C. miltaris* productions by cultivating the *C. militaris* on a range of cereals in Jars within the Laboratory. In this cultivation used brown rice as main nutrients besides barley, sorghum and mung bean powder in Jars as substrates to aid mushroom fruiting body. The objective of this project was to identify the best substrate formulas for optimal cultivation of *C. militaris* **[5].** Furthermore, environmental factors such as pH, temperature, and oxygen levels are important factors that influence the metabolic processes of cordycepin biosynthesis **[6].**

In an attempt to overcome these drawbacks, the current work is centered on the optimization of the growth medium and substrate ratio in order to maximize cordycepin production. Through the examination of the impacts of different carbon and nitrogen sources, as well as other components of the medium, we seek to identify the optimal conditions for increasing cordycepin yield. This research is expected to provide valuable insights into themedium engineering strategies that can be employed for large-scale production of cordycepin, facilitating its commercialization for therapeutic use **[7].**

Research gap identified

Most of the work exists on either cultivation optimization or extraction efficiency, but very few studies combine both cultivation optimization and extraction.

This project fills the gap by:

Modifying the cultivation media to enhance the growth of fruiting body

Using UAE method on a commercial fruiting body to test cordycepin extraction performance.

This project intended to fill the gap by integration of both to enhance cordycepin production.

CHAPTER - 2

LITERATURE REVIEW

C. militaris is a highly beneficial ascomycetous fungus known in Chinese call Bei Dong Chong Xia Cao of Bei Chaong Cao, is a species that commonly parasitizes larva (pupae) of lepidopteran insects and producing fruiting bodies on their insect hosts. *C. militaris is well* known as edible mushroom and one of the significant medicinal mushrooms in the world particularly in Asian countries. Due to its bioactive compounds such as cordycepin, adenosine and polysaccharides, etc., the cordycepin (3'-deoxyadenosine) known as the first bioactive compound extracted from *C. militaris and* used to treat antibacterial, antifungal, antitumor/ antileukemic, antiviral, and immunomodulation activities **[7].** The polysaccharide is mostly used for drugs and food supplement. Both polysaccharide and adenosine that present in *C. militaris* (≥2.5%) and (≥ 0.055%), respectively have been described as quality standards for fruiting bodies by the Chinese Ministry of Health **[8].**

The *C. militaris production* can be acquired through liquid and solid cultivation. According to **[9]**, in commercial field, the fruiting bodies of *C. militaria have* been successfully grown on cereal **substrates** and silkworm pupae. To cultivated *C. militaris* the good sources used in solid state fermentation are cereals such as millet, rye, rice, brown rice, bean powder, com grains, cotton seed hulls, sorghum, corn cobs, jowar, wheat, sunflower floral discs etc. **[4].**

According to current studied, *C. militaris* can grow well on solid fermentation and broth fermentation but not *Cordyceps sinensis*. The production of *C. militaris* that available in world market was successfully cultivated from fruiting bodies. The sufficient carbon and nitrogen sources were provided to support the growth of fruiting bodies. The greatly substrates used for *C. militaris* cultivation were cottonseed coats, wheat grains, bean powder, corn grain, corn cobs, millet, and sorghum. In commercial field applied rice 3 with other organic cereals for *C. militaris* cultivation for high cordycepin production **[9].**

2.1 Bioactive compounds and medical benefits contained in *C. militaris*

The Bioactive compounds and medical benefits of *C. militaris* are composed of cordycepin, adenosine, ergosterol peroxide, fibrinolytic enzyme, xanthophylls, polysaccharides, and antibacterial effect of *C. militaris* **[8].**

(a) Cordycepin

Cordycepin **(Fig. 2.1)**is a bioactive component that synthesis firstly from *C. militaris*. It plays important functions over antimicrobial, antitumor, antimetastatic, immunomodulating, and insecticidal problems. Cordycepin isknown as the significances medical properties with recognition of the importance such as stopped the Ribonucleic acid (RNA) and Deoxyribonucleic acid (DNA) synthesis. It's also recommended for a Phase I and II stage drug over refractory Acute Lymphoblastic Leukemia. The cordycepin was extracted with ethanol was more efficiency than others as methanol. The cordycepin need the temperature of extraction 45-65 °c. The extraction could be done in additional to 30°C at room temperature. The structure formula of Cordycepin known as 3 '-deoxyadenosine. To obtained the Cordycepin a variety of extraction procedures were used including pressurized extraction, Soxhlet extraction and reflux extraction. In order to increasing the cordycepin production and preventing thermal excess the Ultrasonic-assisted (UAE) **[10,57,58].**

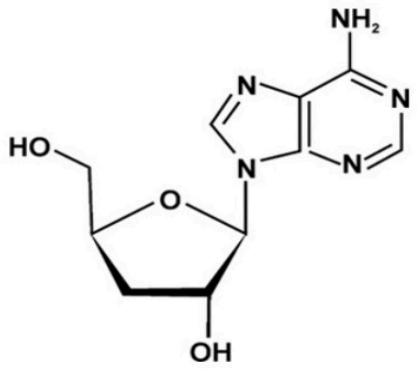


Fig. 2.1: Structure of Cordycepin

(b) Adenosine

Adenosine **(Fig. 2.2)** is one of the bioactive compounds presented in Cordyceps mushroom. It functions of a cytoprotectant (tissue damage inhibition) and used to treat anti-inflammatory, anti-cancer and antioxidant problems. Adenosine that obtained from *C. militaris* was less amount than in *Cordycepssinensis*. The adenosine also listed in biochemical process as potential activity and some used as pharmaceutical goods including anti-inflammatory and

anticonvulsant activities, it might also be recommended for chronic heart failure and tissue damage inhibitions **[10].**

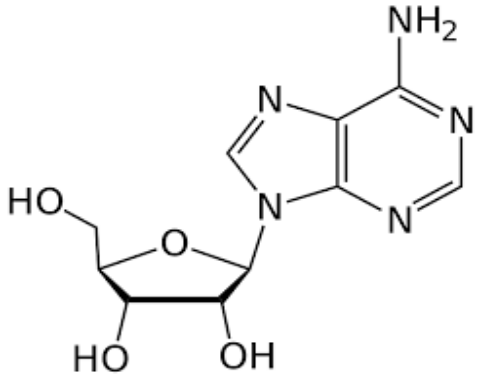


Fig. 2.2: Structure of Adenosine

c) Ergosterol peroxide

Ergosterol peroxide is well known for antitumor sterols that is isolated from edible mushroom (*Ganoderma lucidum* and *Cordyceps sinensis*), it exhibits antimycobacterial, trypanocidal and antineoplastic activities. It is also known for its role in maintaining cell integrity and has been associated with various biological activities, including anti-inflammatory and anticancer effects. In the context of *C. militaris* ergosterol peroxide is one of the secondary metabolites that contributes to the mushroom's pharmacological value alongside cordycepin, adenosine, and polysaccharides. Although present in smaller quantities compared to cordycepin. Its synergistic effect with other compounds may enhance the overall therapeutic potential of *C. militaris* as a functional food and medicinal resource **[22].**

d) Fibrinolytic enzymes

Fibrinolytic enzymes is a bioactive element isolated from *C. militaris*. Fibrinolytic enzymes effect by researches indicated that it has fibrin activity redefined from *C. militaris* and it provides to fibrin pathways modification.

e) Xanthophylls

Xanthophylls is also a bioactive element of *C. militaris* fruiting bodies was reported included Cordyceps carotenoids. Cordyceps carotenoids is potential active compound contained in *C. militaris*. It has been identified that several xanthophylls in the fruiting bodies of *C. militaris*,

including lutein, zeaxanthin, carotene and lycopene. These compounds not only impart color but also play roles in protecting cells against oxidative stress. *C. militaris* produces water-soluble carotenoids and may be more bioavailable. The presence of these xanthophylls and cordyxanthins emphasizes the potential of *C. militaris* as a source of natural antioxidants, that can play a helpful role in designing functional foods or nutraceuticals formulated to neutralize oxidative stress-related conditions **[18].**

f) Polysaccharides

Polysaccharides are complex carbohydrates made up of long chains of the units of Monosaccharide are bonded with glycosidic linkages **[8].** *C. militaris* polysaccharides have high structural elaboration, evolving both the intracellular and the extracellular types. These macromolecules typically consists of heteropolysaccharides composed of various monosaccharides such as glucose, mannose, and galactose **[23].** *C. militaris* polysaccharides have been found to have very strong antioxidant activities. The activities are attributed to their capacity of scavenging free radicals and augumenting the body's endogenous antioxidant defense systems. These are very important in preventing oxidative stress-related disorders and ageing processes **[24].** *C. militaris* polysaccharides immunomodulatory effects has been well evidenced before. They have stimulated the proliferation of lymphocytes, increased macrophage phagocytic activity, and controlled cytokine production, example interleukins and interferons. These are pattern recognition interferons. These are pattern recognition receptors. Besides antioxidant and immunomodulatory activities, *C. militaris* polysaccharides display anti-inflammatory, anti-tumor, and hypoglycemic activities **[25].**

2.2 The Nutrient components of *C. militaris*

To produce *C. militaris* throughout the solid cultivation required the nutrients to supporting the growth of fruiting bodies growth, the composition of nutrients present in *C. militaris* cultivation such as carbohydrates (CHO), lipids (fats), proteins, vitamins, minerals, and water. The fruiting bodies are mostly rich in protein content and hence a good diet source supplement. They have some unavoidable vitamins like B1 (thiamine), B2 (riboflavin), B12, and Vitamin E, which are used in most physiological activities **[14].** *C. militaris* also contain some mineral compounds such as potassium, calcium, magnesium, iron, zinc, which assist enzymatic activities and immune health. The beta-glucans and polysachharides of *C. militaris* is credited for its antioxidant and immunomodulatory actions. Its high content of

composition, *C. militaris* not only a highly potent medicinal mushroom but also a nutraceuticals and functional foods development **[11].**

2.3 Biosynthesis of Cordycepin

Production of cordycepin (3'-deoxyadenosine) in the Cordyceps fungi, particularly in *C. militaris*, encompasses several enzymatic processes starting from purine metabolism. Cordycepin is a modified derivative of the nucleoside adenosine, where the 3'-hydroxyl group is replaced by a hydrogen atom. Below is a simplified outline of its biosynthetic pathway **[20]:**

The biosynthesis of cordycepin begins with adenosine, which is the standard purine nucleoside. Adenosine is synthesized through the purine nucleotide biosynthesis pathway. The key precursor in this pathway is inosine monophosphate (IMP), which is converted to AMP (adenosine monophosphate) by the enzyme AMP deaminase. AMP is then converted into adenosine, which serves as the substrate for cordycepin biosynthesis [10].

The most significant change in the cordycepin biosynthesis is the removal of the 3'-hydroxyl group of the ribose sugar of adenosine. It is catalyzed by the enzyme cordycepin synthetase (also known as adenosine deaminase incertain instances). Cordycepin synthetase converts adenosine to cordycepin by dehydroxylating the 3'-position of the ribose, replacing the hydroxyl group (-OH) with a hydrogen atom.

Cordycepin, or 3'-deoxyadenosine, is the end product of this biosynthetic pathway. It is structurally similar to adenosine, but with the modification at the 3' carbon of the sugar moiety. This structural change is what gives cordycepin its biological activity and makes it distinct from adenosine.

Cordycepin is then incorporated into the fungal biomass or secreted, depending on the species of Cordyceps, where it can exert various biological effects, including anti-inflammatory, anti-cancer, and antimicrobial activities **[1, 30].**

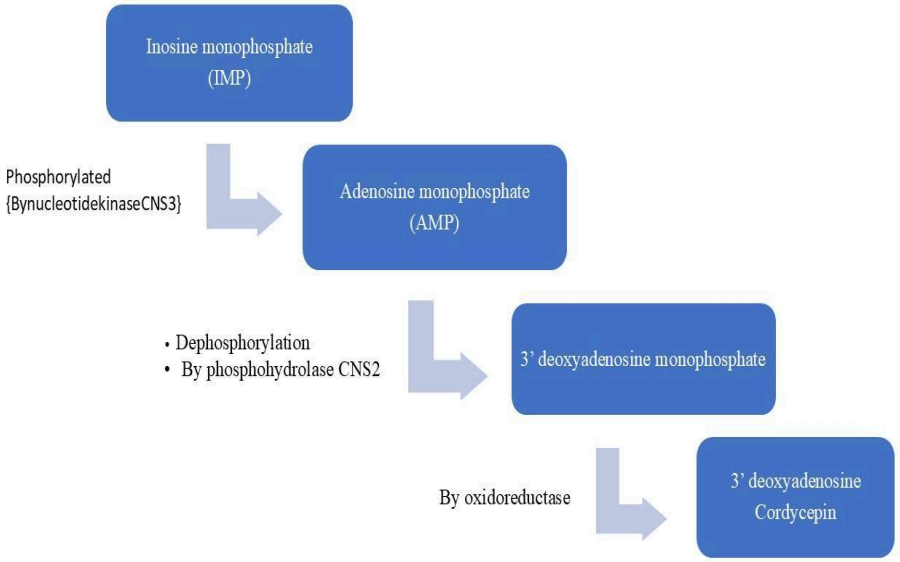


Fig. 2.3: Biosynthetic pathway of Cordycepin

To maximize Cordycepin production through genetic engineering and synthetic biological methods **[20].** Overexpression of the Cs gene, introducing additional copies of Cs into Cordyceps species, can enhance the production of Cordycepin may increase the yield of Cordycepin synthetase**[26].**Metabolic engineering: Redirecting the flow of metabolic intermediates towards cordycepin biosynthesis, eg, overexpressing enzymes that increase the pool of adenosine or AMP, can enhance the availability of precursors for cordycepin synthesis**[3].** Apart from engineering the fungal species itself, synthetic biological methods can be employed to transfer the CNS genes into other microbial hosts, such as yeast, bacteria, to yield Cordycepin in a more controlled environment **[7,39].**

Biosynthesis of cordycepin involves a complex network of molecules beyond the primary precursors and enzymes. Molecules influencing cordycepin biosynthesis, such as Pentostatin, is co-produced with cordycepin in *C. militaris* and play a protective role by inhibiting adenosine deaminase, thereby preventing the degradation of cordycepin. This co-production ensures the stability and accumulation of cordycepin within the fungal cells **[27].** Purine nucleotides (AMP, ADP, ATP) serve as essential precursors in the cordycepin biosynthetic pathway. The conversion processes involving these molecules are crucial for the formation of cordycepin. In an alternative biosynthetic route, RNA degradation leads to the formation of 2',3'-Cyclic AMP and then to cordycepin **[26].** The Cns1-Cns4 gene cluster encodes the enzymes directly involved in the conversion of adenosine to cordycepin. Their coordinated expression is vital for efficient cordycepin biosynthesis. 5'-Nucleotidase facilitates the

dephosphorylation steps necessary for the formation of cordycepin from its nucleotide precursors **[36].**

2.4 Inducers included in Cordycepin Production

Carbon sources such as glucose, Starch or Sucrose, Maltose, Fructose, and Nitrogen sources such ammonium sources are included in Cordycepin production. Carbon sources are one of the most critical components of the culture medium for the biosynthesis of cordycepin, as they serve both as an energy source and as precursors for metabolite production**[2].** Glucose is consistently reported as the most effective carbon source for cordycepin production. It promotes rapid fungal growth and efficient precursor availability for nucleoside biosynthesis. Excess glucose may lead to catabolite repression, so optimized concentrations (typically 20 – 40 g/l) are used. Nitrogen source plays an important role in regulating the metabolic pathways in Cordycepin **[4].** Whereas Ammonium sulphate is also commonly used to enhance the biosynthesis of secondary metabolites like Cordycepin. Yeast extract is rich in amino acids and vitamins which helps in the production of Cordycepin.

Generally its pH and Temperature affect fungal growth, enzyme activity, and metabolite biosynthesis. *C. militaris* grows best and produces higher amounts of Cordycepin in slightly acidic to neutral conditions. Studies show that at pH 6.0, cordycepin production is generally high due to optimal enzyme activity and good fungal morphology and at below pH 5.5 or above 7.5 cordycepin production tends to decrease due to suboptimal enzyme function and stress to the organism. *C. militaris* is a mesophilic fungus, it thrives at moderate temperatures. Optical production of cordycepin is typically observed around 20ºC - 25ºC. Temperature above 28ºC may inhibit growth and reduce metabolite synthesis, while below 18ºC slows down fungal metabolism **[10].** Oxygen is a vital factor that significantly influences the growth, morphology, and secondary metabolite production in *C. militaris*, including cordycepin. As an aerobic fungus *C. militaris* requires sufficient oxygen for efficient metabolism and biosynthesis of cordycepin, which is derived from the biosynthesis of cordycepin, which is derived from the purine metabolic pathway **[7].** High oxygen availability generally enhances cordycepin yield. However too high oxygen levels can cause shear stress that damages the mycelia, reducing productivity. Also, Oxygen availability depends on substrate porosity and surface area **[11].**

The production of cordycepin can be significantly enhanced by the addition of certain inducing compounds or chemical elicitors. These compounds act by stimulating metabolic

pathways, upregulating biosynthetic genes, or creating mild stress conditions that trigger increased secondary metabolite synthesis. Inducing compounds like Adenosine, Methionine, glycine, Citric acid, Surfactants, Metal ions. These all can significantly enhance cordycepin production in *C. militaris*. Thes inducers stimulate the biosynthetic pathways either by acting as precursors, enzyme cofactors or by triggering stress induced secondary metabolism. Optimizing the type, timing, and concentration of these inducers is crucial for achieving maximum cordycepin yield**[31].**

To enhance Cordycepin production, various fermentation strategies have been developed like Submerged fermentation, Solid state fermentation etc. Out of these Solid-state fermentation offers a more natural and economical approach, especially for food-based applications. Fungus is grown on slid substrates with limited free water. It has so many advantages like having low-cost substrates, mimics natural growth conditions, simpler and energy efficient, suitable for functional foods. Substrates used as Corn cob Particles, Barley, Soybeans, Mung beans**[29].**

2.5 Factors influencing Cordycepin stability / Product Compatibility

Cordycepin, a bioactive compound derived from *C. militaris*, exhibits various pharmacological activities. However, its stability is influenced by several factors like pH stability, Cordycepin stability depends on pH. It remains stable in slightly acidic conditions (approximately pH 4-6), which is favorable for oral supplements. However, under alkaline conditions, cordycepin becomes more susceptible to degradation due to hydrolysis. So, maintaining a mildly acidic pH in formulations is crucial to ensure cordycepin's stability. Light also increases bioactive substances at low energy costs in culturing fruiting bodies of *C. militaris*. Cordycepin is thermolabile, meaning it is sensitive to high temperatures. Exposure to temperatures above 60°C leads to its degradation. Low temperature processing methods, such as freeze-drying, are preferred during extraction and formulation to preserve cordycepin's integrity. Thermal sterilization processes in probiotic or beverages products must be carefully designed to avoid compromising cordycepin content **[41].**

While cordycepin primarily degrades through deamination or hydrolysis, oxidative stress can also lead to its breakdown in solution or formulation. Incorporating natural antioxidants, such as ascorbic acid (Vitamin C) or polyphenols can enhance the chemical stability of cordycepin by preventing free radical-mediated degradation **[11].** This is particularly important in multi-ingredient formulations or those stored under light exposure or at room temperature.

When used in functional food products or supplements containing probiotics, cordycepin's compatibility with live microbial cultures must be evaluated **[22].**

2.6 Cereal grains utilized as solid substrates for *C. militaris*cultivation

Thailand is one of the major producers of agricultural products. Agricultural activities depend on most of the Thai population is similar to that, they cultivate a large number of cereals and use those cereals in various fields such as wheat, barley, mungbeans, rice, maize, sorghum, millets, etc. These cereal grains are significant sources of nutrients like energy, carbohydrates, protein, fiber, and macronutrients. They are useful for human consumption, mushroom cultivation, etc. Rice is one of these that becomes a principal food in farming activities**[29].**

(a) Brown Rice

Brown rice is a form of rice that is rich in nutrition and is highly found in the germ or bran layers of Brown Rice. Brown rice can be split into two parts: germinated and ungerminated. The findings indicated that protein, fat, and dietary fibers in ungerminated brown rice were found to increase enormously after germination such as 8.93%, 2.24%, and 4.13% respectively**[21].**

(b) Corncob

Corncobs are a renewable and economic material that has been growing interest for their wide range of uses in agriculture, industry, and biotechnology. Composition – Lignocellulosic materials such ascellulose, hemicelluloses, and lignin. Nutrient content – Nitrogen, Phosphorus, and Potassium. Due to their particle size and composition, they can be used for solid-state fermentation (SSF) and offer an organized environment for the growth of fungi. It exhibited equivalent or better performance in terms of the yield of the fruiting bodies and the content of bioactive compounds**[4].**Corncob particles, a waste product, not only offers environment and economic concerns such as cost-effective substrate but also aids waste reduction. It also serves as an ingredient in various foods and beverages, such as in the production of corn syrup and starch**[5].**

(c) Barley

Barley is one of the grains that are well suited to growing regardless of the conditions in an environment. The major components in barley (dry matter) consisting of 60% starch, 20%

dietary fiber, and 10% protein. The protein in barley functions as reserve nitrogen. The ~-Amylase is major role of nitrogen storage in the growing of the grain and starch-hydrolyzing enzyme in the case of germination**[43].**

(d) Mung beans

Mung bean is one of the legumes that consume mostly in Asian countries such as China, India, Bangladesh, Pakistan, etc. Mung bean are most important sources of protein. Components present in mung bean such as protein, fat, carbohydrates, vitamin B 1 and B2, carotene, niacin, folic acid, minerals, calcium, phosphorus, iron, etc. The mung bean acquired the nitrogen from soil mineral nitrogen as primary nitrogen required for its plant development. The profile of vitamin in mung beanincludes thiamine, riboflavin, niacin, pantothenic acid, and nicotinic acid**[29].**

Table:2.1 Interaction of Cordycepin with Dairy components and its potential benefits

Dairy Components	**Cordycepin Interaction**	**Potential Benefits**	**References**
Probiotics (Lactobacillus, Bifidobacterium)	May enhance beneficial bacteria with inhibition of unwanted ones.	Immune enhancer, gut health, improved digestion	**[11]**
Milk proteins (Casein, whey)	Potential carrier for enhance bioavailability.	Muscle repair, endurance, antifatigue benefits.	**[61]**
Calcium and Vitamin D	Might enhance calcium intake.	Stronge bones, Dec. Osteoporosis risk.	**[14]**
Lactose (Milk Sugar)	Could enhance metabolism of glucose.	Potential control diabetes.	**[13]**
Fatty acids (Omega – 3 in dairy)	Anti-inflammatory activity improved	Enhance brain health, Dec. oxidative stress	**[11]**

2.7 Applications

2.7.1 Cancer Prevention and Treatment: Cordycepin has shown promise as an anti-cancer agent. It can trigger apoptosis (programmed cell death) in different cancer cells and suppress their proliferation by disrupting cancer-related signaling pathways. It has been researched for its ability to treat cancers like breast, lung, liver, and colon cancer. Preventive use: It can also help prevent cancer by suppressing metastasis (spread of cancer cells) and inhibiting the growth of new blood vessels that supply tumors (angiogenesis) **[32,38].**

2.7.2 Anti-Inflammatory and Immune Modulation - Inflammatory Conditions: Because of its anti-inflammatory effects, cordycepin is also investigated as a therapy for chronic inflammatory diseases, including arthritis, asthma, and inflammatory bowel disease (IBD). Immune System Support: It is employed to enhance immune function, specifically in enhancing the activity of immune cells such as macrophages, T cells, and natural killer (NK) cells. This makes it is beneficial in treating infections and potentially enhancing the body's defense against autoimmune diseases**[44].**

2.7.3 Antimicrobial and Antiviral Applications - Antibacterial and Antifungal: Cordycepin has indicated antibacterial activity, but for example against drug-resistant strains of bacteria such as Staphylococcus aureus. It is antifungal and has proved to be useful in the treatment of fungal infection. Antiviral Use: Cordycepin has been found to be effective against certain viral infections like influenza and HIV. It inhibits viral replication, therefore it is potent candidate in the treatment of viral infections.Neuroprotection and Cognitive Improvement - Alzheimer’s and Neurodegenerative Diseases: Cordycepin has also tested for its neuroprotective activity, especially against Alzheimer’s disease, Parkinson’s disease, and other neurodegenerative diseases. It may be prevent brain cells from oxidative stress and inflammation, improving cognitive capacity and may be slowing the progression of these diseases **[33].** Cognitive Function: Cordycepin is believed to improve learning and memory, so it is an excellent candidate for improving mental function, particularly in the aging populations.

2.7.4 Anti-Aging - Prevention of aging: Antioxidant effect of cordycepin is one of the reasons help towards its possible anti-aging potential. It may be potentially protect cells from oxidative stress, which accelerates the aging process. It also enhances DNA repair and controls genes involvement in aging leading to longer life span. Skin Care: As a result of its antioxidant and anti-inflammatory activity, cordycepin is studied for use in skincare products,

in which it is utilized to reverse wrinkles, enhance skin elasticity, and provide protection against skin aging**[42].**

2.7.5 Cardiovascular Health - Cardio protection: Cordycepin was discovered to protect the heart from ischemia (lack of blood supply) and oxidative stress, both of which can damage heart tissue. It could lower the chances of heart attacks and stroke by enhance blood flow and preventing clotting **[33].** Regulation of blood Pressure and Cholesterol Regulation: Cordycepin reduces the blood pressure and normalizes the cholesterol levels thereby resulting in overall cardiovascular wellbeing. Metabolic Health and Weight Management - Diabetes Management: Cordycepin was also found to enhance insulin sensitivity and regulate glucose metabolism. This makes it a therapeutic agent for type 2 diabetes control and preventing insulin resistance. Weight Loss: It can assist in weight control by regulating fat metabolism and promoting fat burning. Cordycepin has been studied for its ability to minimize fat accumulation and optimize metabolic activity, and can be use to cure obesity. Physical performance and fatigue improvement – boosting of energy: Cordycepin possesses anti-fatigue properties and is extensively used for the promotion of energy and the combat of fatigue. It is believed to enhance mitochondrial performance and stamina by optimizing oxygen use in the body. Athletic Performance: Due to its ability to improve endurance and preventing fatigue, cordycepin is also employed by sportsmen occasionally as an enhancer of performance. It can enhance endurance and recovery after strenuous physical activity **[34].**

2.7.6 Antithrombotic and Antiplatelet uses - Blood Clot Prevention: Cordycepin has antithrombotic and antiplatelet effects, and thus is utilized for prevention and thrombosis. This can reduce the risk of such conditions as stroke, deep vein thrombosis (DVT), and heart attack. Dietary Supplements - Functional Foods: Because of its numerous health benefits, cordycepin comes in food supplements, herbal supplements, and functional foods. It is marketed for increase energy, enhance immune function, enhance skin, and managing chronic conditions like diabetes and hypertension. Cosmetic Industry - Anti-aging and Skin Care Products: Being anti-inflammatory, antioxidant, and anti-aging in nature, cordycepin is used in cosmetics and skincare products. It prevents skin damage from oxidative stress and UV radiation and enhances healthy skin aging **[42].**

2.8 Reported Cordycepin–enriched products

2.8.1 Fruity Selenium-enriched C. *militaris* Yogurt

Selenium-enriched Yogurt contains selenium, which is a trace element that has antioxidant properties and is used in supporting immune function. It involves incorporating protoplasts of *C. militaris*, a fungus that is recognized for its medicinal application, into yogurt. This integration ensures that the yogurt benefits from added health advantages. For enhancing taste and consumer acceptability, the yogurt is infused with natural fruit flavor, giving in a product that is both nutritious and flavorful **[35].**

2.8.2 Korean ginseng chicken soup

The quality and functional enhancement of documented Korean ginseng chicken soup by enzymolysis pretreatment with *C. militaris* mushroom extract. Ginseng, the core functional component, is known for its antioxidant, anti-inflammatory, and immune-boosting properties **[59].**

2.8.3 Cordyceps Milk

Cordycepin milk retains all the health benefits of milk and further add its properties by bioactive factors of *C. militaris,* such as Cordycepin and Polysaccharides: Enhance immune function and improve metabolism. SOD enzymes and Amino acids – Decreased oxidative stress, enhance antiaging and support muscle recovery. Vitamins and trace elements – Maintain heart health, increase oxygen uptake and boost energy levels **[61].**

2.8.4 Sugar free flavored yogurt

Sugar free Cordyceps yogurt is a blend of *C. militaris* and yogurt and uses aspartame and acesulfame as alternatives to sucrose. This functional yogurt maintains the flavor and health benefits of conventional yogurt while Cordyceps.

It provides affordable means to broaden the use of *C. militaris* while enhancing the yogurt industry **[60].**

CHAPTER – 3

Materials and Methods

3.1 Chemicals and Materials

The culture of *C. militaris* was obtained from DMR, Solan maintained under sterile conditions. Various agro-based substrates were employed as solid media, including brown rice, corncobs, Soybeans, mungbeans, and barley purchased from the local market. For the preparation of liquid culture and fermentation media, the following nutrient components were used like glucose(dextrose), peptone of Qualigens Laboratory India, yeast extract of Himedia Laboratory India, magnesium sulphate, potassium dihydrogen phosphate of Merck Life Science Private Limited, and Thiamine/Neurobion of Proctor and Gamble Health Limited by local market. Distilled water was used as the solvent throughout the media preparation. Ethanol 100% was used as primary solvent for the extraction of cordycepin from dried fungal biomass fermentation.

Several laboratory instruments were also used during the cultivation, extraction and processing. Incubation of liquid culture was carried out in a shaking incubator of Thermo Scientific India to promote mycelium growth. Solid-state cultures were incubated under static conditions in a stationary incubator of Thermo Scientific India at 20°C to support the development of fruiting bodies. All the media, substrates, the culture vessels were sterilized in an Autoclave of Sanyo Technology India at 121°C and 15 psi prior to inoculation. A Sonicator of Citizen Industries was used to disrupt fungal cell walls and enhance the release of cordycepin through ultrasonic treatment. After which, Whatman filter paper was used for filtration of the biomass extract. The ethanol extract was concentrated using a rotary evaporator of Heidolph Instruments, which removed the solvent under reduced pressure and controlled temperature conditions. All the procedures were performed under aseptic conditions using standard laboratory equipment.

3.2 Method of Cultivation

Media Preparation

First, the seed cultures of *C. militaris* mycelia strain was collected from DMR, Solan.

Subculturing of *C. militaris* - Weigh 0.78 gm of PDA powder and dissolve in 20 ml of distilled water per test tube. Close the test tubes with cotton plugs and then Autoclave it. After autoclaving, place them at an angle of 30˚- 45˚The entire medium will solidify as a slanted surface, increasing the area for fungal growth. Allow the slants to fully solidify at room temperature.

Inoculation of *C. militaris* - Inside laminar air flow near a flame with a sterilized inoculation loop transfer a small piece of actively growing mycelium onto the surface of the PDA slant. Incubate at 22˚-25˚C in the dark for 7-14 days until the mycelium fully colonizes the slant.

Liquid spawn preparation - Liquid spawn of *C. militaris* was prepared using a nutrient rich medium to support fast mycelium growth. Liquid spawn is prepared by dissolving dextrose (2g), yeast extract (1g), and peptone (1g) in 100 ml of distilled water which is prepared in 250 ml flask, then sterilization is done at 121°C and 15 psi using an autoclave. After cooling to room temperature, the sterile medium was inoculated with actively growing mycelium of *C. militaris* under aseptic conditions inside a laminar airflow. The inoculated flask were then placed in a shaking incubator at 25°C with continuous agitation at 120 rpm for 5-6 days. The incubation allowed for the development of mycelium, which later was used as liquid inoculum (spawn) for both solid and liquid fermentation experiments **[29].**

Table 3.1 Ingredients of liquid spawn

Ingredients	Concentration (g/ml)
Dextrose	**2**
Yeast extract	**1**
Peptone	**1**

Liquid media preparation - The liquid medium consists of glucose, peptone, yeast extract, magnesium sulphate, potassium dihydrogen phosphate, and multivitamins, dissolved in distilled water in 1000 ml flask. The components were completely mixed and the pH was adjusted to 6 **[30].**

Table 3.2 Ingredients of liquid media

Ingredients	Concentration (g/ml)
Peptone	2.5
Magnesium sulphate	0.25
Multivitamins	0.01
Yeast extract	1.5
Glucose	15
Potassium dihydrogen phosphate	1

3.3 Solid Substrate Media Preparation

C. militaris was cultivated on the medium using different substrates as stated below:

3.3.1 Rice

Took 30g of rice as a substrate and added 35 mL of nutrient solution to moisten the substrate. The jars were then covered and autoclaved at 121°C to ensure sterility. Upon cooling, jars were aseptically inoculated with 3-4 ml of actively growing liquid spawn of *C. militaris*. The inoculated jars were topped with Whatman filter paper to allow for gas exchange while maintaining sterility. The jars were subsequently incubated at 20°C in the dark for 10 days to promote vegetative development of mycelium. After this, the jars were then transferred into light regimes and incubated once more for further 6-7 days for further development of fruiting bodies. **[21,43].**

3.3.2 Corncob

Used 30g of Corncob as a substrate and added 35 mL of nutrient solution to moisturize the substrate. The jars were autoclaved and sealed at 121°C to sterilized them. After cooling, jars were aseptically inoculated with 3-4 ml of actively growing liquid *C. militaris* were aseptically inoculated into the jars. The inoculated jars were covered with Whatman filter paper for gas exchange while maintaining them under sterile conditions. The jars were then incubated in the dark at 20°C for 10 days to induce vegetative mycelial growth. After this, the jars were then put under light conditions and incubated for another 6-7 days to provide support for growth of fruiting bodies. The color of the mycelium will turn pink or orange. Then we will provide 800 – 1000 lux light daily for 10 – 12 hours. Then, within the next 20 –

25 days, mature fruit bodies will be formed. Then harvesting of mushrooms from Jars, which can be then used for the production of its bioactive compound Cordycepin. Fruit bodies obtained were dried to constant weight at 50˚C **[43].**

3.3.3 Barley

Took 30g of Barley as a substrate and added 35 mL of nutrient solution to moisten the substrate. The jars were then covered and autoclaved at 121°C to ensure sterility. Once cooled, jars were aseptically inoculated with 3-4 ml of actively growing *C. militaris* liquid spawn. The inoculated jars were covered with Whatman filter paper to allow gas exchange while maintained sterile. The jars were then incubated in darkness at 20°C for 10 days to support vegetative development of mycelium. After this, the jars were shifted to light conditions and incubated for an additional 6-7 days to favor the development of fruiting bodies. **[43].**

3.3.4 Soybean

Used 30g of Soybean as a substrate and supplemented with 35 mL of nutrient solution to hydrate the substrate. The jars were autoclaved and closed at 121°Cin a sterile state for storage. Once cooled, jars were aseptically inoculated with 3-4 ml of actively growing liquid of *C. militaris* was aseptically inoculated into the jars. The inoculated jars were covered with Whatman filter paper for gas exchange while preserving them free from contamination. The jars were then incubated in dark conditions at 20°C for 10 days for vegetative mycelial growth. After this, the jars were then supplied with light conditions and incubated for the rest of the 6-7 days for enhancement in development of fruiting bodies **[43].**

3.3.5 Mungbean

Used 30g of Mungbean as a substrate and added 35 mL of nutrient solution to moisten the substrate. The jars were then closed and autoclaved at 121°C to ensure sterility. Then cooled, jars were aseptically inoculated with 3-4 ml of actively growing liquid *C. militaris* in jars. The inoculated jars were covered with Whatman filter paper to allow exchange of gases while maintained sterile. The jars were subsequently incubated in the dark at 20°C for 10 days to induce vegetative growth of mycelium. After this, the jars were then kept under light conditions and incubated for another 6-7 days to facilitate the development of fruiting bodies **[43].**

3.4 Extraction of Cordycepin

The extraction of cordycepin from *C. militaris* was carried out using ultrasonic assisted extraction, due to its efficiency, simplicity, and ability to preserve the integrity of thermolabile compounds with 100% ethanol, for disrupting fungal cell walls and releasing intracellular metabolites **[10].**

Method for the extraction of cordycepin

C. militaris fruiting bodies cultured on supplemented substrates were treated with ethanol extraction to determine cordycepin yield. 0.9g sample was used and 18ml of 100% ethanol was added, have a solid to liquid ratio of 1:20 (g/ml). The mixture was placed in an ultrasonic bath at 70°C, with an ultrasonic frequency of 56 kHz, for 5 hours to facilitate the extraction process. After sonication, the extract was filtered using Whatman filter paper to remove solid residues**[58].**

CHAPTER - 4

RESULTS AND DISCUSSION

4.1 Cultivation of *C. militaris*

Subculturing of *C. militaris* was carried out to maintain fungal viability and ensure consistent performance for experimental inoculation. After actively growing mycelium was transferred from the master culture to the slants, the subcultured strains retained morphological characteristics including dense, white to pale orange-colored mycelium with a cottony texture as shown in **fig: 4 (b).**These results are in agreement with previous finding that emphasized the importance of maintaining healthy stock cultures on peptone-supplemented PDA slants to support stable growth and subsequent fruiting body production **[43].** Liquid spawn of *C. militaris* was prepared using a nutrient-rich medium to support fast mycelium growth by dissolving dextrose (2g), yeast extract (1g), and peptone (1g) in 100 ml of distilled water. Then, after incubation of the inoculated flask, visible mycelial growth appeared within 48 hours, forming a fluffy mycelium throughout the liquid medium. The uniform dispersion of fungal biomass shows the efficient oxygen transfer and nutrient utilization, which is important for liquid spawn development. This reflets the medium's capacity to support fungal metabolism and biomass accumulation. In the study, where *C. militaris* strain was cultured in a glucose-peptone-yeast extract medium and exhibited mycelium proliferation under shaking conditions at 25°C. The mycelium mass in that study reached its maximum within 7 days, shows that optimized liquid media plays a crucial role in preparing high quality spawn for cultivation. The consistency between our observation and the cited literature shows the effectiveness of subculturing and liquid media optimization as foundational step to ensure successful fruiting bodies and metabolite production **[43].**

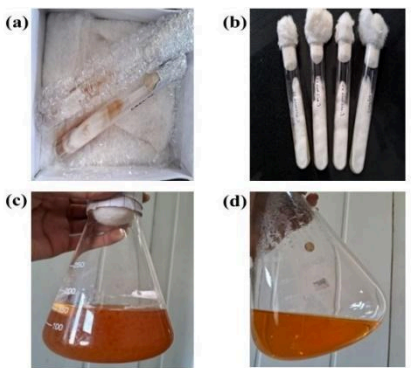
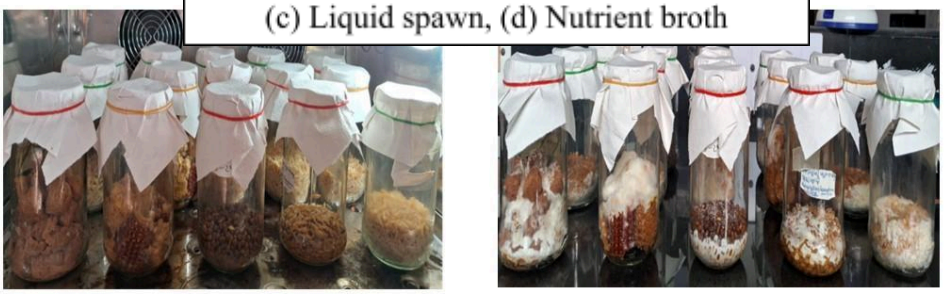


Fig. 4.1 (a), (b): Subculturing of *C. militaris,*

(c) Liquid spawn, (d) Nutrient broth

(e)



(g)

(h)

Fig. 4.1: (e) Inoculated jars by culture of *C. militaris* (f): After 6-7 days of incubation (g) Color of mycelium turned into orange (h): Fruiting body starts appearing after 20-25 days

(i)

(j)

(k)

Fig. 4.1 (i): Matured fruiting bodies appeared after 44 days in Corncob

(j) Length of fruiting body 3.5cm

The growth of the fruiting body of *C. militaris* was evaluated on five different agro-based solid substrates: corncob, rice, soybean, mungbean, and barley. Among these, Corncob was the only substrate that supported complete fruiting body cultivation. On corncob, *C. militaris* shows early mycelial colonization within 3 days, with the primordial occurrence observed by day 12, and mature fruiting bodies appeared by day 44. The fruiting body appeared a healthy orange coloration with distinct strip-like structures reaching an average length of 3.5cm. This is probably due to the relatively higher content of starch in Corncob, which may have provided a more balanced nutrient environment for the development of fruiting bodies.

In contrast, all the other substrates failed to produce fruiting bodies. Mycelium growth was observed within 3-4 days, and primordial occurrence was delayed until day 12-4, with no fruiting body maturation by day 46. The fungal colonies on rice and soybean exhibited dense orange-colored mycelium, while mungbean and barley showed lighter orange to yellow pigmentation, indicating suboptimal nutrition for full fruiting body development. The inability of these substrates to support mature fruiting bodies could be linked to factors such as insufficient carbohydrate complexity, poor aeration, or moisture retention issues. The comparative results are summarized in Table 4.1, which shows that corncob is a significantly superior substrate for supporting the complete lifecycle of *C. militaris* under the conditions tested. These findings suggest that substrate composition plays a crucial role in the physiological development of *C. militaris* **[48].**

Table 4.1 Effect of different substrates on growth and development of *C. militaris*

Substrates	**Mycelium growth time (days)**	**Premordial occurrence time (days)**	**Fruitbody mature time(days)**	**Fruit body appearance**
Corncobs	3	12	44	Orange color strips 3.5 cm
Rice	3	12	44	Orange color mycelium
Soybean	4	14	46	Orange color mycelium
Mungbean	4	14	46	Light orange or yellow color appearance
Barley	4	14	46	Orange color appearance

These results are consistent with the finding reported in the literature cited here found that corncobs were one of the most suitable substrates for the cultivation of *C. militaris*. Their study shows high yield and enhanced concentration of key bioactive compounds like Cordycepin and Adenosine. The current findings show the importance of substrate composition in the physiological development of *C. militaris* and show corncob as a cost-effective and sustainable substrate for enhancing fruiting body yield and bioactive compound like cordycepin production **[40,48].**

4.2 Harvesting and Processing of Fruiting Bodies

Once the fruiting bodies of *C. militaris* reached maturity, they were carefully harvested by hand using sterilized forceps. The fresh fruiting bodies were weighed to record their fresh biomass and then subjected to drying. Drying was carried out in a hot air oven at a temperature of 50°C ,ensuring the removal of moisture without degrading heat-sensitive bioactive compounds like Cordycepin.

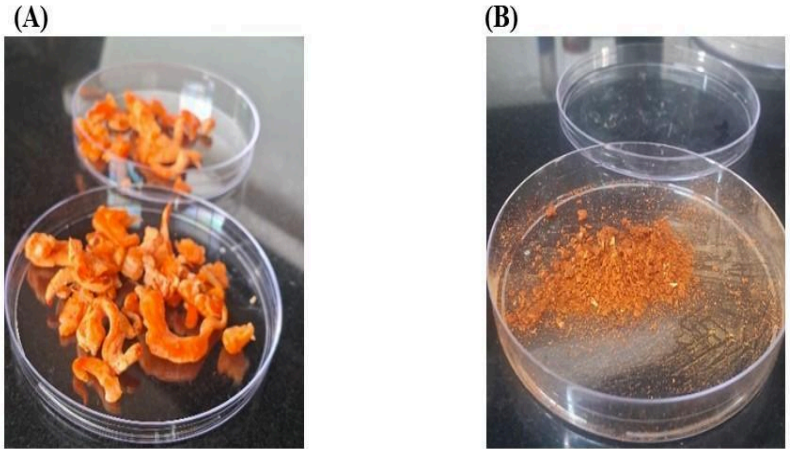


Fig. 4.2 (A): Harvesting matured fruiting bodies of weight 12.7g

(B) Powdered form after drying in a hot air oven at 50°C weight of 0.9g

The results of the present study, which shows the impact of substrates on the growth and fruiting body of *C. militaris*, are partially consistent with findings reported by **[48]**. As their study reported that they used 40g dry substrate per bottle with supplementation, while we use 30g per jar, possibly without any added nutrient like wheat or rice bran.so, the size of their fruiting body was 5-6 cm. This could lead to lower yield of fruiting bodies and also smaller size **[56].**

4.3 Extraction of Cordycepin from *C. militaris*

The extraction of cordycepin from *C. militaris* was carried out using ultrasonic assisted extraction with 100% ethanol, for disrupting fungal cell walls and releasing intracellular metabolites.

Extraction of Cordycepin from Fruiting Body

Commercial sample of *C. militaris* was also done by ultrasonic ethanol extraction procedure. 1.8 gm sample of fruiting body was mixed with 100% ethanol (36ml) and processed in Ultrasonic bath and set temperature at 70°C and Ultrasonic frequency to 56KHz for 5 hours. Then filtered the extract using Whatman filter paper and collect the extract. Using Rotary evaporator to remove solvents like ethanol and concentrate the extract of cordycepin. Extraction obtained after removal of solvent is 0.107g of crude extract **[10 ,58].**

Cordycepin from *C. militaris* can be done by using a variety of solvent-based techniques. Among these ethanol extraction, particularly when combined with ultrasonic-assisted extraction, is widely used due to its efficiency and ability to preserve the thermolabile compound. Ethanol is often preferred over methanol or water due to its polarity, low toxicity and cordycepin solubilization **[58].**

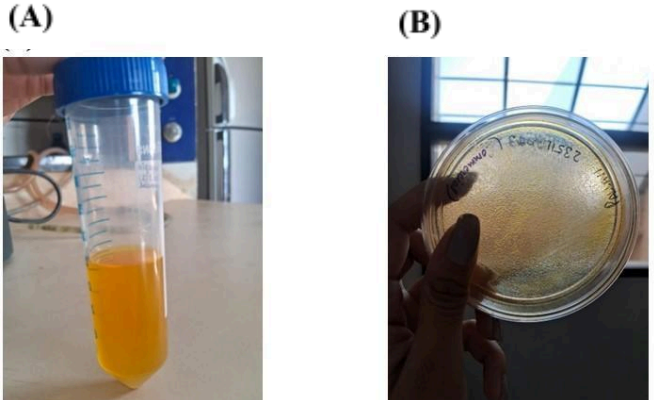


Fig.4.3(A): Filtered extract, (B) After ethanol removal weight of extract (0.107gm = 107mg)

4.4Calculation of Yield

Sample = 1.8 g

Solvent = 36 ml ethanol (100%)

Extraction obtained after Rotary evaporation =0.107gm = 107mg

Yield = 0.107gm/1.8gm×100

= 5.94 %

The observed yield aligns well with findings from the literature cited below, who reported that cordycepin content was highest in 100% ethanol extracts of fruiting bodies of *C. militaris*, even though lower ethanol concentration gave higher crude extract yield. Their study highlighted that although 20% ethanol provided the highest total extraction mass (13.5%), the cordycepin concentration peaked at 15.2 mg/g when 100% ethanol was used for the effectiveness of absolute ethanol for extraction of cordycepin. They shows that ultrasonic-assisted extraction greatly enhances cordycepin release by disrupting fungal cell walls and preserving the compound under moderate heat. The result shows that combination of ultrasonic extraction at 70°C and absolute ethanol is effective for recovering cordycepin from *C. militaris*. The 5.94% yield achieved in this study falls well within the expected range reported in the literature which is 3-7% for ethanol based method **[58].**

CONCLUSION

Under this project, the research investigated a combined method of increasing dual approach to enhancing cordycepin yield from *C. militari s*through the combination of substrate optimization during cultivation and Ultrasound-assisted extraction techniques. A modified substrate was employed for growing fruiting body under controlled conditions. Even the cultured sample could not be extracted because oftechnical difficulties, the growth data itself confirmed that substrate engineering has a positive impact on biological yield. To confirm the extraction process, a commercial sample of *C. militaris* was given Ultrasonic-assisted ethanol extraction, then solvent removal via Rotary evaporation.

In general, the work demonstrates that even partial results can also provide valuable information, and it sets the ground for the future work that will directly link optimized conditions of cultivation conditions towards improved cordycepin yield.

FUTURE SCOPE

This research opens up several promising directions for future work. One of the next steps would be to scale up cultivation trials using various media combinations to further enhance biomass and compound yield. Advanced analytical techniques such as High Performance Liquid Chromatography (HPLC) should be used to quantitatively assess Cordycepin content across different substrates and growth conditions. The bioactive extracts obtained can be screened for key pharmacological properties like anti-inflammatory activity to validate their therapeutic potential. These findings could support the development of innovative functional food or nutraceutical products enriched with Cordycepin extract, for commercial applications in health and wellness industries.

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