BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, September 2024. Vol. **21**(3), p. 839-849

Future of Scarlet Caterpillar Club Fungus: A Review on Molecular Strategies for Cordycepin Enhancement

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http://dx.doi.org/10.13005/bbra/3268

(Received: 03 May 2024; accepted: 08 July 2024)

Cordycepin, a novel nucleoside derived from the Scarlet Caterpillar Club fungus, has gained considerable attention for its broad spectrum of biological properties profitable in the medicinal sector. Despite being significant in the pharmaceutical and cosmeceutical sectors, its difficult cultivating techniques make it hard to produce in large quantities. Its commercial potential depends on large-scale production improvements. In the laboratory, cordycepin can be synthesized via chemical and biosynthetic pathways. Although chemical synthesis offers precise control, mass manufacturing is not economically viable. Thus, several biosynthetic pathways are modified for a comprehensive investigation of variables, particularly enzyme function and metabolic flux, that affect the synthesis of cordycepin. For production at a larger scale, several fermentation techniques are employed, out of which, the submerged or liquid fermentation proves to be more economical to achieve greater yield. Another key approach that significantly influences cordycepin production aims to improve culture conditions, like temperature, pH, vitamin concentrations, carbon, and nitrogen sources. Diverse substrate selections can point to improvement in the growth of fungus. Production also varies with the effect of different sources of nitrogen and carbon or carbon/nitrogen ratios on Cordyceps militaris growth and glucose and dextrose are the most efficient carbon sources for the growth of C. militaris, while peptone is primarily used as a nitrogen source. The large-scale production of cordycepin can also employ corn steep liquor hydrolysate, a secondary metabolite from several industries, as a nitrogen source thus increasing cordycepin yield and is economical. Protoplast fusion has a significant role in achieving higher cordycepin production from C. militaris, when its protoplast was fused within the same species or different species of he genus with a better mycelial growth. strain selection using modern molecular techniques is also a significant variable for improving yield, cordycepin synthesis is now better understood owing to the use of omics technologies and upregulating the genes that regulate the cordycepin biosynthesis pathway in C. militaris. This article presents an in-depth discussion of the molecular approaches used to increase the production of cordycepin.

Keywords: Biosynthesis; Cordycepin; Culture optimization; Genetic Engineering; Omics Technologies; Molecular Strategies; Synthetic Biology.

An ancient Chinese medicinal fungus, Cordyceps contains a variety of bioactive components, like cordycepin, adenosine, polysaccharide, and ergosterol[1]. Cordycepin, an adenosine derivative also known as 3'-deoxyadenosine, is a natural chemical majorly found in some species of the genus Cordyceps and is one of the most important active components.

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It offers several pharmacological activities, including anti-cancer, anti-inflammatory, anti-viral properties [1,2] and many more. It has a wide range of mode of action as in case of cancer it inhibits the proliferation of the cancerous tissue by inducing apoptosis and in cases of viral diseases, it inhibits the replication process of the viruses that can cause the disease [1,2]. It also is proven to play a crucial role in cosmeceutical in anti-photoaging and anti-pigmentation [1]. However, the market price of this essential secondary metabolite has increased exponentially, thus the production of cordycepin has been achieved from the extracts of the fruiting body of *C. militaris* by solid state or liquid fermentation where the production period is comparatively shorter, and the conditions can be controlled [3]. In the below review we are going to discuss about media optimization to produce high cordycepin in which we optimize temperature, pH, nitrogen source and carbon source and strain improvement techniques like irradiation with physical mutagens like UV rays, protoplast fusion and chemical mutagenesis. We are also going to discuss the modern strain improvement approaches involving transcriptomics, proteomics, and metabolomics.

Synthesis of Cordycepin

Various techniques used to synthesise cordycepin involve chemical, microbial, in vitro processes and biosynthesis [1]. The biosynthesis of cordycepin, in *Cordyceps militaris* is affected by elements, like the components of the operational factors and gene expression profiles [1]

Biosynthesis

The process of creating cordycepin through biosynthesis includes refining both masuds techniques along with modifying *C. militaris* to boost the production of cordycepin.

Liquid Fermentation

The promising method, for increasing cordycepin production is through fermentation of *C. militaris* although productivity levels remain relatively low at present. The content of Cordycepin (COR) varies among strains making it crucial to prioritize screening for strains with COR content in COR production. Various optimization techniques have been explored to enhance the COR content in fermentation. These optimization options include screening for strains with COR content, ion beam irradiation, integrating additives in the medium, optimising composition, using two-stage shaking static culture methods, and using LED irradiation [1]

The goal was to reduce costs by around half and increase output by over 30% using a new reasonably priced liquid fermentation combination. Furthermore, by combining reflux extraction and resin adsorption, cordycepin was purified to 95.23%. Pupa powder and wheat bran were used in the liquid fermentation medium [4].

In another approach various methods such as single factor experiments Placket–Burman design, central composite design and response surface methodology were used to enhance the fermentation medium and culture conditions for boosting cordycepin content. The study focused on seven variables, with temperature, yeast extract and tryptone concentrations singled out as factors influencing cordycepin yield. After optimization the ideal medium consisted of 9.00 g/L of yeast extract and 17.10 g/L of tryptone resulting in a yield of 7.35 g/L in a 5 L fermenter, under culture conditions [5].

Optimizing cordycepin production, in *Cordyceps militaris* batch fermentation involved a two phase strategy that combined shaking and static fermentation. The growth of hyphae and conidiophores plays a role in enhancing cordycepin production with growth and production rates varying across the shake and static fermentation phases. By implementing the two step shake fermentation method there was an increase in *C. militaris* cell concentration, during the shaking phase, followed by successful cordycepin production during the static phase resulting in a final yield of 2.62 g/L [6].

Solid Fermentation

The process of producing COR through solid state fermentation (SSF) typically takes longer compared to fermentation lasting around 30 days. Despite the duration SSF can yield the fruiting body for obtaining COR. In contrast, to fermentation the COR content is primarily detected in the fruiting body when produced through SSF. Various optimizations have been applied to enhance COR content, such, as utilizing UVB and LED radiation with wavelengths adjusting pH levels controlling temperature, managing culture time and optimizing the composition of substrates .

The researchers experimented with

materials, like brown rice, millet, corn, wheat and glutinous rice to see how well they supported the growth of fruiting bodies and the yield of cordycepin in solid state fermentation. They also studied the impact of carbon and nitrogen sources, mineral salts and several growth factors, on fruiting body development and cordycepin production using experimental methods [7]

Enhancement of cordycepin using media optimizing techniques

Culture condition optimization

Strain selection

To extract cordycepin in high amounts, strains like KYL05 can be used which has a high cordycepin productivity. Various other strains were studied to produce cordycepin in high amount with an outstanding mycelial growth like KCTC 6064, KCTC 6862, KSP8 and G81-3[8]. Thus, for commercialization the highest cordycepin producing strain KYL05 is usually mutated using UV radiation and growth were observed on agar after 10-15 days [2,9]. In many studies, media optimization is carried out on the strain KSP8 using solid fermentation on Brown rice medium and Silkworm pupae medium or submerged cultivation in media of different compositions[1].

Media composition optimization

Studies have been done on effects of various carbon sources and carbon/nitrogen ratios on the growth of *Cordyceps militaris* and the production of cordycepin under a varied controlled condition. Upon investigations on the nutrient sources, glucose was observed to be an effective and economic source of carbon cordycepin production, although similar or even better growth patterns can be observed when culture is carried out in the presence of sucrose or lactose as the carbon source for several strains of C.militaris[10].With an increase in the initial glucose concentration, there was a surge in the growth of *C. militaris*. An increase in the initial glucose content resulted in the increase in the cell growth of cordyceps[11]. Furthermore, nitrogen sources in the media also plays a crucial role in the growth of the fungus. Amongst the various organic and inorganic sources of nitrogen like yeast extract, peptone, ammonia, usage of peptone resulted in higher production of cordycepin[11]. However, in a comparative study, similar enhanced growth was observed by using casein hydrolysate (CH). The result showed that the cordycepin concentration increased with an increase in the CH concentration. In other studies, the use of Corn Steep Liquor Hydrolysate (CSLH) resulted in a lesser peptone consumption, and higher cordycepin yield, thus in future CSLH holds the potential to serve as a nitrogen source or *C. militaris* cultivation[12].

pH optimization, temp optimization

Along with the media composition, other physical factors like temperature, pH, light significantly affect the growth of the fungus. As most fungus proliferate effectively at a temperature range of 20-25 °C, a study on Japanese *C. militaris* showed results of enhanced mycelial growth at temperature range of 15-20°C[13]. However for strains collected from other regions, the optimal growth was achieved at 25°C[14]. Thus, maximum growth was achieved at 25°C where the cordycepin production were found to be approximately 2 folds higher than other temperatures. The maximum yield of cordycepin was achieved when the culture was maintained at a pH of 6 and a shaking condition of 150 rpm[8].

Genetic approaches for strain improvement of cordyceps

Strain enhancement is the process of boosting the qualities of microorganisms, commonly bacteria, fungi, or yeast, for a specific purpose in industries or biotechnology. This enhancement might focus on genetic, metabolic, or physiological adjustments aimed at amplifying the microorganism's productivity, efficiency, or adaptability to conditions or duties [15]. The process often entails employing methods like genetic manipulation, inducing mutations, or selectively breeding to cultivate traits beneficial for the intended use, such as augmenting the yield of a desired output, enhancing resistance to environmental pressures, or optimizing nutrient utilization. It holds significant importance across various domains like pharmaceuticals, agriculture, food manufacturing, and environmental management [16].

For the strain improvement of cordyceps there are many methods like mutagenesis, protoplast fusion, regeneration, and transformation [17]. Many research [17] has been done for the improvement in the quality of cordyceps and the quantity of the products obtained from it . Some of the methods are discussed as follows

Transformation System

This technique is used to focus the functions of genes related to various biological processes. The process involves introducing foreign DNA into the cells of host organism, ensuring the transferred DNA is stably maintained and replicated, and ultimately expressing the desired trait as shown in Figure 1. This approach facilitates the manipulation of an organism's genome, linking laboratory DNA studies with real-world biological results. Numerous techniques are employed to create transformation systems, such as electroporation, polyethylene glycol (PEG) treatment, integration via protoplasts using restriction enzymes, gene transfer facilitated by *Agrobacterium tumefaciens*, particle bombardment, DNA uptake by cells treated with lithium acetate, protoplast transformation, and nitrate reductase transformation[17].

In order to identify functional genes in *C. militaris*, researchers developed and refined a system for transformation by *Agrobacterium tumefaciens*. They also observed PEG-mediated transformation which involved isolating *C. militaris* protoplast using enzyme mixtures like Yatalase, Usukizyme, and lysozyme. During transformation, the efficiency of transformants varied from 30 to 600 per 1 × 105 conidia when *C. militaris* and *A. tumefaciens* cells were cultured together in the presence of acetosyringone for 2 days [18]. The pH level was discovered to impact transformation efficiency, with the highest number of resistant colonies achieved at pH 5.5. Consequently, a cultivation medium with pH 5.5 was deemed optimal for developing the *C. militaris* ATMT system [19].

To validate stable vector integration in transformants, researchers conducted mitotic stability analysis after monosporic purification. Later by southern bloting and then analysing the data of transformants showed vector hybridization, with integration potentially being directed or random. Random integrations might occur due to low similarity between the genes of the two integrated genomes.

Protoplast Fusion

Protoplast fusion has emerged as a strategy to overcome the limitations associated with traditional breeding methods, enabling the transfer of genes across barriers. This technique plays a pivotal role in enhancing strains through genetic recombination, fostering the development of hybrid strains in both micro and macro fungi[20]. By allowing the amalgamation of genes from disparate organisms, protoplast fusion facilitates the creation of strains possessing desired traits. Notably, it has been particularly effective in augmenting mushroom strains by the help of certain enzymes and osmotic stabilizers.

In a specific study, protoplast fusion was conducted using two distinct species of cordyceps (CM1 and CM2) to generate a hybrid [2].While the resulting hybrids exhibited high biomass production, their stability across subsequent generations was not sustained, prompting further investigation. Protoplast fusion becomes necessary when two mating types are unavailable[21].

This technique can be applied intra-specifically, inter-specifically, inter-generically, and even inter-heterogenerically. For example, protoplasts from two different species within the Cordyceps genus, namely Cm029B and CS, were fused, thus resulting hybrid strains were looked via RAPD-PCR methods[17].

To isolate protoplasts from the mycelia of fungus, an specific technique based on Hashiba [22] was utilized, involving lytic enzyme treatment followed by protoplast separation via filtration. The isolated protoplasts were then washed and suspended in an osmotic stabilizer solution. Protoplast fusion was induced using polyethylene glycol (PEG 30%), CaCl2 (0.05 M), and glycine (0.05 M) at pH 7.5. The fused protoplasts were cultured on regeneration medium under controlled conditions to observe and record colony growth and fusion rates [17].

Characterization studies, including analysis of mycelial growth, hyphal size, and isozyme patterns, were conducted to evaluate the hybrids. Furthermore, RAPD-PCR analysis confirmed the stable formation of hybrids between *Cordyceps militaris* and *Cordyceps sinensis.*

UV irradiation

Ultraviolet (UV) irradiation involves exposing an object or substance to electromagnetic radiation within the ultraviolet spectrum, which has a very short wavelengths and high energy, typically ranging from about 10 nanometers (nm) to 400 nm [23]. This method is utilized for strain improvement by inducing mutations in the wild type organism.

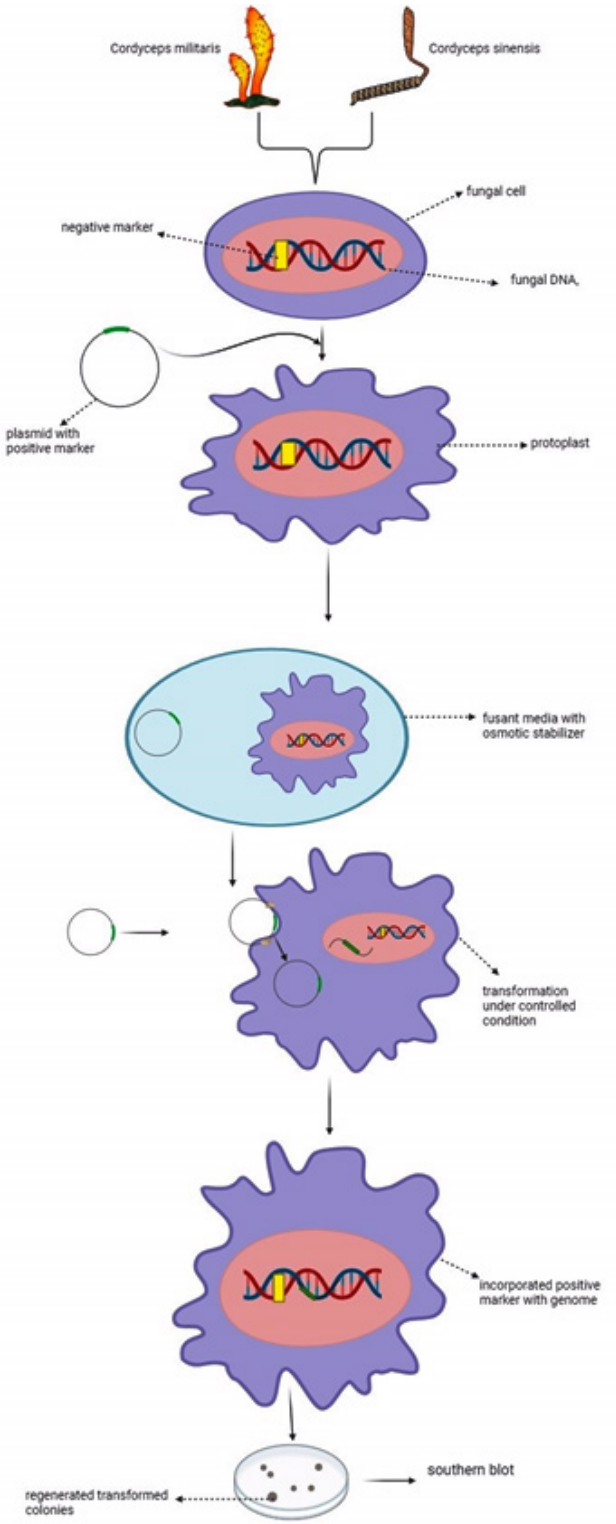

Fig. 1. Transformation method for Cordycepin using Cordyceps militaris and Cordyceps sinensis

To induce sporulation, strains were cultured on CMA plates at a temperature of 20ºC and further subcultured. After subculturing, a conidial suspension was made in 0.85% NaCl solution with 0.1% Tween 80. This suspension solution was further introduced with sodium nitrate at a concentration of 500ìg/ml and exposed to UV irradiation for 80 minutes under a UV lamp positioned 50 cm away. Following irradiation, the suspension was incubated at a temperature of 20ºC for 60 minutes in darkness [24]. Subsequently, 0.1 ml of the suspension was plated on solidified PMP medium to inhibit fungal colony growth. The culture was then incubated at 20ºC for 6 days until colonies became visible. Any isolates displaying altered characteristics after incubation were recognised as mutants.

After incubation for 6-8 days, only an average of five viable colonies were observed. These colonies were then inoculated into 150ml of PMP broth and incubated for 10 days at 20°C. Following incubation, the cultures were plated on CMA plates. From each parental strain, MTCC-3936 and CCM-1 were selected for further analysis. Notably, the CCM-1 isolate exhibited a change in colony color from white to black. Additionally, MTCC-3936 displayed slow growth, indicating a mutation. However, this method resulted in a reduced harvest of live spores and decreased cell viability. Moreover, with repeated subculturing, it was noted that mutant colonies gradually lost their distinctive characteristics and viability over time[24].

In another study two hybrids are taken and irradiated with UV, the two mutants from each hybrid were selected and analyzed for further development [2] giving sub-lethal doses of UV radiation increasing the production of cordycepin [25] which is stable through many subcultures.

Chemical mutagenesis

A variety of chemicals or compounds can be used as mutagens to alter the structure of genome permanetly. These mutagens are of mainly three types -base modifiers, base analogues, intercalating agents.

From the previous fusion hybrids, CMF5U1 is taken and further mutated with ethidium bromide by incorporating the ethidium bromide and another batch which are just physically exposed which leads to high biomass production [2]. Cordycepin production from wild type increased many folds in suspension culture rather than using a normal substrate [26].

Table 1. Optimization Strategies and Cordycepin Yields

Strategy	Main Optimization Methods	Result in Cordycepin Yield	Reference
Solid Fermentation	Optimization of culture conditions (temperature, pH, substrate composition)	Increase of fruiting body yield to 67.96% (1.73±0.08 g/bottle) and cordycepin content in fruiting body to 63.17% (9.17±0.09 mg/g)	7
Liquid Fermentation	Addition of various growth supplements	Cordycepin content increased 31.8 and 19.7 times using glycine and glutamine amino acids as compared to control	28
Protoplast Fusion	*A. cinnamomea* and *C. militaris* protoplast fusion from different phyla	The yields of adenosine, biomass, cordycepic acid, cordycepin, total polysaccharide, and total triterpenoids in high-outputting fusants were increased up 1.305"50.1563 times in comparison to the initial culturing	29
Proton Beam Irradiation	*C. militaris* mutant G81-3, was obtained by high-energy proton beam irradiation	For the mutant strain, the optimum dose ofadenosine yielded a 30% increase in cordycepin production	30

In another study, the hybrids were stabilized using abrin which is a toxic compound extracted from a plant called rosary pea shows the elevated production of cordycepin [24]. In another study, scientists carried out sexual reproduction between two compatible parental strains, spores were isolated from them which gave rise to eight different mated strains. 32 crosses were made in which the strain (ksp8) showed high cordycepin production. It is concluded that the increase in cordycepin production is due to recombination in the genes [27].

Recent research on cordycepin and related compounds focuses on optimizing yields through various strategies. Table 1 summarizes key strategies, main optimizations, and resulting cordycepin yields. Approaches include solid fermentation, liquid fermentation, protoplast fusion, and proton beam irradiation showing significant yield improvements for commercial and therapeutic applications.

Omics-Guided Strategies for Cordycepin Enhancement

Omics technologies, like transcriptomics, proteomics, and metabolomics, are making it simpler to understand biological systems from tissue to the molecular level providing a detailed understanding of gene expression and regulation, protein interactions, and metabolite profiles [31].

Transcriptomic studies can be very helpful for understanding into the transcriptional level of the genes about how they are getting involved in biosynthetic pathways, as well as regulatory mechanisms for Cordycepin production [32]. Differential gene expression analysis can also tell us the regulatory genes and factors, like transcription factors and signalling pathways, that modify Cordycepin production concerning environmental factors and media availability [28].

Proteomic approaches, like one-dimensional or two-dimensional gel electrophoresis coupled with the use of mass spectrometry (MS), are extensively utilised for identifying proteins and enzymes in fungi and other closely related species [33]. To find the protein expression between wild-type and genetically engineered strains a Comparative proteomic study can be done and this can also be done to identify potential protein molecules related to raised Cordycepin production [34].

A comprehensive study of small-molecule metabolites in biological systems can be also done via Metabolomics to gain knowledge of metabolic pathways and understand cellular metabolism[35]. Metabolomic techniques provide a significant understanding of metabolic flux as well as the regulation of Cordycepin biosynthesis pathways [36].

The cointegration of all the omics technology mainly transcriptomic, proteomic, and metabolomic databases makes it possible for systems-level modelling of Cordycepin biosynthetic pathways, finding critical regulating points and metabolic constraints [35]. By understanding the molecular mechanisms guiding Cordycepin synthesis, omics-guided advancements can also help us to understand and apply targeted genetic and metabolic engineering strategies that will enhance Cordycepin yield [36].

Economic Importance

Cordycepin, a compound found in certain fungi species like Cordyceps, has long been revered in traditional medicine across cultures. In traditional Chinese medicine (TCM), it's been utilized for centuries to enhance vitality, boost immunity, and improve respiratory function[37]. Similarly, in Tibetan medicine, known as "yartsa gunbu," or 'summer grass winter worm' Cordycepin is esteemed as a potent tonic for treating respiratory issues and enhancing overall well-being[38]. Beyond its medicinal use, cordycepin holds a significant place in folklore, particularly in the Himalayan regions where it's referred to as "Himalayan Gold". This rich tapestry of traditional use and folklore underscores cordycepin's enduring cultural significance and its continued exploration in modern science for its pharmacological potential[39].

The global market for cordycepin is experiencing robust growth, driven by rising consumer awareness about its health benefits and increasing demand for natural remedies. According to market research reports, the demand for cordycepin is expected to continue growing steadily in the coming years, propelled by factors such as the growing aging population, rising prevalence of chronic diseases, and shifting consumer preferences towards natural and organic products[39]. Pharmaceutical companies are exploring the development of cordycepin-based drugs and formulations for various therapeutic purposes. Clinical trials are underway to evaluate the safety and efficacy of cordycepin in humans,

with promising results reported in preclinical studies. The successful commercialization of cordycepin-based pharmaceuticals could have a significant 16 economic impact, not only in terms of revenue generation but also in improving public health outcomes[40]. In addition to standalone supplements, cordycepin is also being incorporated into functional foods and beverages to enhance their nutritional value and health-promoting properties. Products like energy bars, protein shakes, and herbal teas fortified with cordycepin are gaining popularity among fitness enthusiasts and consumers seeking holistic approaches to wellness. The growing demand for functional foods and beverages presents lucrative opportunities for manufacturers to capitalize on cordycepin's market potential[41].

Challenges and Future Directions

In the fields of medical research and biotechnology, cordycepin has huge potential. However, there are several challenges but there are mainly technological limitations and regulatory issues that limit its comprehensive application of cordycepin.

First of all, achieving superior and better yield is still a major challenge as there are many technological limitations in the current production processes [42]. Researchers in a study bring focus to the ongoing issue of poor yield, which limits Cordycepin's commercial viability. This is correlated with a complex biosynthetic pathway, which is characterised by multiple stages of enzymatic reactions and regulatory complications [1]. Several attempts have been there to understand and modify this pathway for increased production but still, these experience significant challenges. Furthermore, Cordycepin biosynthesis is dependent upon specific substrate availability, which further complicates the manufacturing process.

Along with technical hurdles, regulatory challenges dominate more. Regulatory agencies impose strict limitations based on the safety and efficacy of bioactive compounds. Regulatory approval of enhanced Cordycepin products requires proof of their safety profile and therapeutic efficacy [43]. Further complicating regulatory matters of intellectual property is a major challenge, which is especially significant when talking about genetic engineering methods and synthetic biology strategies [44]. The management of intellectual property is a major challenge for researchers and commercialisation initiatives. Moreover, regulatory norms are different in different countries which additionally adds a logistical issue, requiring special attention to production procedures, product quality, and labelling specifications.

Dealing with such challenges requires an interdisciplinary approach. Modern genetic engineering techniques, like Cas9 technology, offer an opportunity to enable precise alterations that improve biosynthetic pathways and thus improving yield [45]. The use of novel bioprocess strategies like fed-batch and continuous fermentation systems can be used to improve the culture conditions for increased Cordycepin production [46]. Optimising substrate supply and rerouting metabolic flux towards Cordycepin biosynthesis pathways are also some strategies to improve overall production efficiency through metabolic engineering.

Looking ahead, future research offers potential solutions. Exploration of some new biosynthetic pathways and enzyme cascades in heterologous hosts could open new avenues for enhancing production efficiency. Integrating multi-omics approaches, such as transcriptomics, proteomics, and metabolomics analyses, promises comprehensive insights into molecular mechanisms governing Cordycepin biosynthesis, identifying potential metabolic engineering targets. The development of sustainable production methods, such as fermentation technology using renewable feedstocks or bioremediation approaches, holds great promise in minimizing environmental impact and resource consumption. Furthermore, establishing standardised protocols for the production and quality control of enhanced Cordycepin formulations across diverse therapeutic areas and are essential steps towards realising the full potential of Cordycepin in medicine and biotechnology.

CONCLUSION

In summary, Cordycepin, derived from the medicinal fungus Cordyceps, displays a wide range of pharmacological effects spanning from anti-inflammatory to anti-cancer properties. Traditionally sourced from the fruiting body of *Cordyceps militaris* through solid-state or liquid fermentation, contemporary methods strive to

enhance production through media composition adjustments and strain enhancement techniques. Further research can explore the scalability of the optimized media formulation for industrial scale production. Additionally, investigations into the impact of media composition on the production of specific bioactive compounds within *Cordyceps militaris* like cordycepin. Nevertheless, challenges persist, encompassing technological constraints, regulatory complexities, and the management of intellectual property. Addressing these hurdles necessitates interdisciplinary collaboration, utilizing modern genetic engineering tools, refining bioprocesses, and exploring novel biosynthetic pathways. Future research endeavors are geared towards developing sustainable production methods, establishing standardized protocols, and gaining comprehensive understanding of Cordycepin's molecular mechanisms, thereby fostering its broad application in medicine and biotechnology.

ACKNOWLEDGEMENT

We would like to express our sincere gratitude to the department of Molecular Biology and Genetic Engineering of Lovely Professional University for providing us the sources and our mentors Dr. Vikas Sharma and Dr. Shivika Sharma for their invaluable mentorship throughout our work.

Conflict of Interest

There is no conflict of interest.

Funding Source

There is no funding sources.

Author Contribution Statement

The authors confirm contribution to the paper as follows: Conceptualize and design of the review paper: R.K, N.M; Introduction and Enhancement of Cordycepin using media optimisation section:N.M; Synthesis of Cordycepin section,Economic importance and Table 1: R.K.; Genetic approaches for strain improvement of Cordyceps: A.S., S.N.R; Omics-guided strategies, challenges, and future directions: A.C.; Conclusion, Figure 1: A.S;Overall supervision of review paper: Dr. S.S. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

Not Applicable.

Ethics Approval Statement

Not Applicable

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