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# An overview of the cultivation and commercialization of the caterpillar fungus, sited in the Tibetan Plateau and the Himalayan forests of Bhutan and Nepal

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## Abstract

*Ophiocordyceps sinensis* is a unique entomopathogenic fungus and valuable Chinese medicine resource that has been employed for treating various human conditions. Limited *O. sinensis* in the wild due to over-exploitation has led it to the brink of extinction. This caused a massive disparity between supply and demand, resulting in skyrocketing prices. The dumping of counterfeit products in the market also caused the need for quality control. In this review, effort has been made to understand the development of *O. sinensis* and its life cycle, in which the possible cultivation method can be discussed. Additionally, it also summarizes the analytical method for quality control measures in order to ensure the quality of artificially cultivated O. sinensis are on par or even better than the wild. Furthermore, the commercialization of artificially cultivated Cordyceps is lightly touched. Despite these challenges, research into the cultivation of Ophiocordyceps sinensis continues, as it has the potential to provide a sustainable source of the fungus for medicinal purposes. Some pharmaceutical companies have already developed products containing *Ophiocordyceps sinensis*, and further research may lead to the discovery of new therapeutic applications for the fungus. However, it is important to ensure that the cultivation and commercialization of *Ophiocordyceps sinensis* is done in an ethical and sustainable manner, to avoid further depletion of the wild populations of the fungus.

**Keywords**: Ophiocordyceps sinensis, cordyceps, cultivation, quality control, commercial

## Introduction

The fungus *Cordyceps* spp. is remarkably well known for its medicinal properties as well as potent chemical compounds. In fact, it has been used as remedies for various diseases by local folks of Tibet for over 500 years (Arora, 2015). The word Cordyceps was derived from Greek expression 'kordyl' and Latin phrase 'ceps', meaning 'club' and 'head' , which describing the fungus appearance (Das et al., 2021; Olatunji et al., 2018). There are relatively 500 species of *Cordyceps* spp. identified to date, among which *Ophiocordyceps sinensis* (syn. *Cordyceps sinensis*) being the most expensive due to its rarity (Kunhorm et al., 2019). In addition, it is also the most well-documented Cordyceps species with reports reaching back to the late 1400s from Tibetan doctors (Chen et al., 2013). It is widely known as 'Dong Chong Xia Cao', meaning summer-plant winter-worm in the practice of Traditional Chinese Medicine (TCM) (Arora, 2015).

This caterpillar fungus is distributed to Tibetan Plateau that includes Tibet, Gansu, Qinghai, Sichuan, and Yunnan Provinces in China (Li et al., 2019), as well as Nepal, Bhutan, and India (Panicker, 2017). *O. sinensis* is acknowledge having various bioactive components as well as chemical constituents, contributing to numerous benefits to humankind. The components that have been documented hitherto include cordycepin, modified nucleosides, polysaccharides, and sterols (Chen et al., 2017). These components have shown therapeutic properties in treating conditions such as asthenia, arrythmias, hyperglycemia, hyperlipidemia, hyposexuality, night sweats, and other heart, respiratory, renal, and liver diseases (Lo et al., 2013; Zhou et al., 2009). Besides, it was also reported that *O. sinensis* plays a role in a variety of biological processes, including antipathogenic, insecticidal, anticancer, and neuroprotective properties, as well as protection against ischemia/reperfusion damage (Cao et al., 2020).

The numerous medicinal properties offered have caused increasing demand in the market, making the industry strong and growing. Various products have been commercialized in the market in a variety of forms, such as pills, capsules, energy drinks, powder etc. These products are produced from the caterpillar mushroom, mycelia, or mixture of both or with other medicinal mushroom, which mostly targets improving immune system as well as overall health benefits. Besides, some of the products also offer to improve lungs and kidney function, support healthy vascular systems, and promote better stamina and sexual behavior. Globally, *O. sinensis* alone is anticipated to produce 85–185 tons per year, with additional tonnage contributed by other Cordyceps species (Elkhateeb et al., 2019). The massive marketing of cordyceps has resulted in the dumping of counterfeit products in the market, thus, its

authenticity and quality control are critical. Various chemical makers have been suggested in controlling the quality of the marketed product so that their benefits can be deliver (Wei et al., 2017).

In addition, active commercialization has resulted in over exploitation of wild cordyceps, which indirectly destroyed their natural habitat. To make it worst, the global warming has caused an upward movement of the snow line, which cause further decrease of cordyceps yield (Li et al., 2019). Therefore, the Convention on International Trade in Endangered Species (CITES) Management Authority of China formally designated *O. sinensis* as an endangered species in 2012 (Xu et al., 2016). In consequence, due to the scarcity and harvesting difficulty, the price of wild cordyceps has shoot up approximately USD 15,000 per kilogram (Choda, 2017). This situation has led researchers, especially Chinese and Japanese, to discover alternative solution to these problems. Artificial cultivation is one of the solutions sought by researchers to overcome the issues of excessive excavation of wild sources.

The purpose of this review is to provide a summary of conventional methods of artificial cultivation and their limitations. This review also provides a summary of previous study made on artificial cultivation of *O. sinensis* via fermentation technology of liquid culture and solid culture. The method of quality control in ensuring the artificially cultivated *O. sinensis* is comparable to the wild will also be discussed. Finally, a review of literature regarding the commercialization of artificially cultivated *O. sinensis* will be provided. It is anticipated that this review will provide relevant and practical information to researchers investigating artificial cultivation of *O. sinensis* specifically, or *Cordyceps* spp. in general.

## Development of *Ophiocordyceps sinensis*

### The life cycle of *O. sinensis* host

*O. sinensis* is fungi that parasitize insect larvae as part of their life cycle. Generally, it can grow on all group of insects including ants, bees, black beetles, centipedes, cockroaches, and crickets (Elkhateeb et al., 2019). It frequently demonstrates a high level of host specificity with a wide host range spanning from ten orders of arthropods to truffle-like genus *Elaphomyces* (Yue et al., 2013). However, most species are confined to a single host species or a group of closely related hosts (Xu et al., 2016; Yue et al., 2013). Initially, the fungus was first discovered on *Hepialus armoricanus*, which later placed within *Thitarodes* (Viette 1968) under the order of Lepidoptera (Wang & Yao, 2011). The larvae of *Hepialus* or *Thitarodes* provide *O. sinensis* with nutrients needed for growth, and they are mostly found within Himalayan Plateau as well

as temperate regions of Eastern China (Cannon et al., 2009; Lo et al., 2013). To date, 50 species have been described with 57 taxa identified as possible hosts for *O. sinensis* (Baral et al., 2015; Cannon et al., 2009; Lo et al., 2013).

Generally, the developmental stages of host insect can be divided into four, i.e., egg, larva, pupa, and adult, which consume 3 to 5 years for a complete life cycle (Li et al., 2019). *Thitarodes pui* larvae, for example, develop over a period of 1095–1460 days, with the egg stage lasting 41–47 days, the larval stage 990–1350 days, the pupal stage 35–41 days, and the adult stage 3–8 days (Li et al., 2019; Zou et al., 2012). larval stage lasts seven to nine instars, spending the most of its time eating on subterranean roots (Zou et al., 2012). According to field research, the most host infection by *O. sinensis* occurred while larvae in the $4^{th}$ to $5^{th}$ in stars were shedding old cuticles and creating new ones (Yang et al., 1989; Zhang et al., 2012). These cuticles (procuticle and epicuticle) is a thick layer covering epidermis of the larvae, known as integument (Tuli et al., 2014).

The integuments of insects are made of chitin, proteins, and lipids as well as range of enzymes and phenolic components (Leger et al., 1991; Tuli et al., 2014). The formation of epidermis consists of a single layer of epithelial cells followed by a thick layer of procuticle, which can be divided into two parts of endocuticle (inner soft part) and exocuticle (outer hard part). Epicuticle and wax are known to make up the cuticle's outermost coat (Tuli et al., 2014). These components of integument act as defense mechanism against pathogen, as well as restricts water loss and acts as a link between the insect and its surrounding (Tuli et al., 2014). This makes it challenging for *O. sinensis* as it must penetrate the resilient integument covering to gain entry into the host. However, larvae are susceptible to be infected by *O. sinensis* during shedding, which happened in nature from early to mid-August, which coincides with the discharge of sexual ascospores by *O. sinensis* (Yang et al., 1989; Zhang et al., 2012).

However, *O. sinensis* rarely infected larvae at below $4^{th}$ instar due to their restricted movement and food intake. The same occurrence happened to those at advance stages ($6^{th}$ instar and above) due to their increased resistance (Zhang et al., 2012). Commonly, upon infected, larvae become less active in 6 to 10 days, during which they frequently relocate to 2 to 5 cm beneath the soil surface before dying in 15 to 25 days (Yang et al., 1989). The relocation of larvae beneath soil surface facilitates the formation of the fruiting body and its emergence from the soil the following year (Zhang et al., 2012).

**The mode of *O. sinensis* infection on host**

The fundamental steps in the fungal life cycle, such as the mechanism of infection, subsequent growth, and asexual or sexual development, appear to be coordinated by many signals (Baral, 2017). Ophiocordyceps infective propagules are short-lived and lack survival characteristics such as melanization and significant nutritional resources (Cannon et al., 2009). Besides, they also do not produce large mycelial networks in soil, from which new infective propagules might emerge (Cannon et al., 2009). Therefore, it is presumed that the infection of larvae occurred right upon spore discharge from the fungus stroma, usually in the late autumn. The highest rate of infection occurs in the 3$^{rd}$ to 4$^{th}$ instar or 4$^{th}$ to 5$^{th}$ instar, where the new cuticles replacing the old one (Li et al., 2019; Yang et al., 1989).

The approach of larvae infection can occur in two different manners, i.e., skin infection and intestinal infection (Li et al., 2019). Skin infection begins upon contact of fungus conidia with surface of the larvae, which under ideal conditions, will germinate within a few hours (Tuli et al., 2014). In order to get protection from UV radiation of the environment and reactive oxygen species (ROS), conidia released protective enzymes superoxide dismutase (SOD) and peroxidases (Tuli et al., 2014; Wang et al., 2005). In addition, conidia also produce hydrolytic enzymes such as protease, chitinase, and lipases to dissolve the larvae integument as a mean to gain entry (Tuli et al., 2014). Upon effective penetration, the mycelia network expands rapidly, allowing endotoxins to be delivered to the larval blood vessels (Panicker, 2017).

However, penetration via integument can be difficult due to resilient cuticle that is made of wax and epicuticle (Panicker, 2017), which lead the fungus to intrude via larvae’s mouth when its feeding on the roots of small grasses (Baral, 2017). The invaded fungus then move further to the gut (Hu et al., 2013). The fungus penetrates the larvae's hemocoel, fragmenting into fusiform hyphae, multiplying by yeast-type budding to fill the hemocoel (Li et al., 2019), and concentrates within the lipid reserve of the larvae (Cannon et al., 2009). The larval immune system is suppressed by the fungus's entry into the hemolymph, which leads to the insect's death, particularly through hunger, convulsion, or other physiological or biochemical disturbance induced by the fungus's development (Panicker, 2017; Charnley, 2003).

Soon after, a germ-tube-like structure appears, allowing the fungus to be directed into the interior of the cuticle rather than spreading horizontally just in the cuticular layer (Baral, 2017; Charnley, 2003), forming plate-like structure called penetration plate. Further penetration plate creates secondary hyphae, from which protoplast bodies bud off and travel into hemocoel of the larvae (Tuli et al., 2014). The filamentous mode of fungus invaded internal organs and

tissues of the host by producing numerous toxic secondary metabolites that are insecticidal, causing the larvae paralyzed, dies out, and mummified by the fungal mycelium (Baral, 2017; Tuli et al., 2014). This allows the fungus to survive the upcoming winter (Baral et al., 2015). Before the soil starts to freeze in winter, a small stroma will bud off from the dead larvae's fontanel (Baral et al., 2015; Li et al., 2019). The stroma bud develops upward the next spring, erupting above the soil surface and creating a stalked fruiting body (a sexual, perithecial stroma), where thread-like ascospores are released around July (Zhang et al., 2012). This collided with the flight period and egg-laying of the host moths that occurred on early August. These ascospores can be spread by wind or water and can presumably infect new larvae (Li et al., 2019; Zhang et al., 2012). The stromata may be able to release spores for a long period, and release may be triggered by comparable environmental circumstances like temperature and/or humidity to those that cause the host insects to emerge (Cannon et al., 2009). Figure 1 summarizes the life cycle of host and *O. sinensis*.

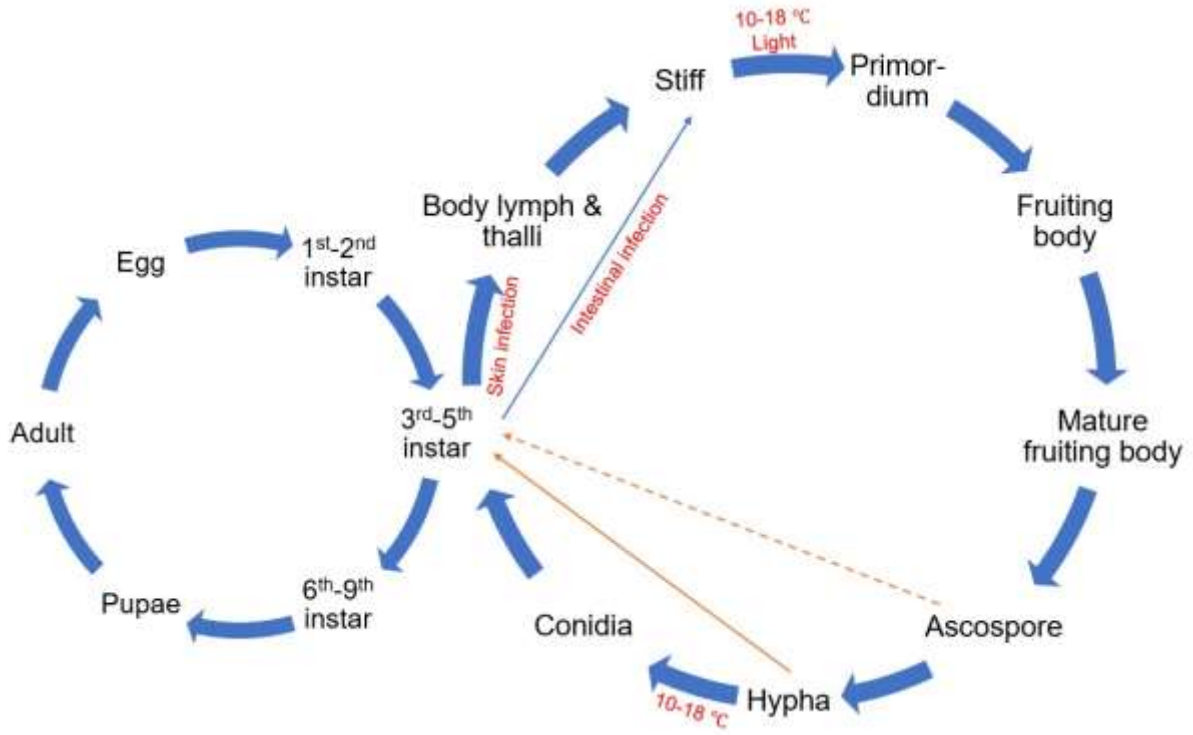


**Figure 1** The cycle of host life and infection of *O. sinensis*.

## Cultivation of *Ophiocordyceps sinensis*

Cordyceps fruiting bodies that develop naturally are scarce and collecting them is a costly operation (Elkhateeb & Daba, 2020). *O. sinensis* are artificially produced and commercialized

for human consumption as well as research and pharmacological purposes, particularly in China and United States (Choda, 2017; Zhou et al., 2009). The cultivation of *O. sinensis* can be achieved via conventional method and fermentation.

**Conventional method**

The conventional method of artificial cultivation of *O. sinensis* is hard to achieve due to difficulty to mimic natural habitat of both host insect and the fungus. Besides, during its lengthy, prolonged developing stages, the wild *O. sinensis* passes through many growth phases in the natural environment, which need unique growth requirements (Choda, 2017). Therefore, in order to successfully artificially cultivate the fungus, few conditions need to be met, including: 1) isolation of fungi; 2) generation of *Hepialus* spp. host larvae; 3) fungal spores' mechanism of infection; and 4) growth environment simulation involving illumination, temperature, pressure, and humidity (Zhou et al., 2014). There are two types of artificial cultivation, namely complete artificial cultivation mode and semi-natural cultivation mode.

**Complete artificial cultivation mode**

The complete artificial cultivation mode applies the whole culture process under artificial conditions, from rearing of host larvae and culturing fungi to infection of larvae with fungi through inoculation. The process takes approximately 2 years for the caterpillar mushroom to arise. This technique provides improvement in larvae survival rate as well as reduce the period of growth as compared to naturally occurred caterpillar mushroom, although it is costly for large-scale production (Zhou et al., 2014). The rate of infection and the primordium induction are also improved through this mode of cultivation. One company in Guandong, China has successfully adopted this cultivation technique for commercialization purpose. The development of this technique took several stages before succeeding.

Firstly, in order to avoid reproductive degeneration and achieve disease-resistant host types, researchers employed germplasm screening and hybrid breeding of the host insect (Li et al., 2016a). Following success, the first stage building of workshops and facilities in a factory to imitate the environment of the Tibetan Plateau in a low altitude zone was constructed in 2007 (Li et al., 2016b). Secondly, preliminary investigation was done for scale up production to determine the suitability of cordyceps growth in the controlled environment, which resulted to subsequent second stage workshop construction in 2010 (Li et al., 2019). Then, the fungus and hosts were analyzed for their morphology and molecular and identified as *O. sinensis* and *Hepialus xiaojinensis* from artificially cultured samples (Wei et al., 2016). The fruiting body

of artificially cultivated *O. sinensis* under controlled environment can also reach sexual maturity by producing perithecium and ascospores (Li et al., 2019), upon which the third stage workshop was established. It is estimated the production of *O. sinensis* will reach 30 tons annually, approximately 20% of the total natural resource (Li et al., 2019).

**Semi-natural cultivation mode**

On the other hand, semi-natural cultivation mode applies the same concept for rearing and infection of larvae, except that the successfully infected larvae are released into the wild to grow at their own pace. The caterpillar-fungus complex formed upon maturity in 3-5 years in the nature. The advantages of this mode is the reduction of cost as well as able to make the best of natural resources (Zhou et al., 2014). The only drawback of this mode is the rate of survival of the infected larvae released to the wild is unstable, since they are exposed to harsh environments such as weather, soil, and food conditions (Li et al., 2006; Zhou et al., 2014). This mode of cultivation has been studied for years, however the adoption to large scale production remains uncertain. Therefore, in order to solve the problems, it is important to study on increasing the production of *O. sinensis* in natural environments by the artificial manipulation of concurrently increasing the number of *Hepialus* larvae and their rate of infection by *O. sinensis* (Li et al., 2019).

One of the concerns studied by researchers for this cultivation mode is the ability of the infected larvae to gain nutrition since it was one of the challenges that determine their survival. A study conducted previously on the diet of Thitarodes larvae in Qinghai-Tibetan Plateau proposed that omnivorous larvae preferred feeding on tender roots of Polygonaceae plants (Lei et al., 2011). Another investigation used stable carbon isotope analysis to look into the diet of Thitarodes larvae in Tibet's Sejila Mountain and discovered for the first time that humic compounds in habitat soils might be an alternate food source (Chen et al., 2009). Further study on the potential of humic compounds as alternative food source for Thitarodes larvae has been investigated. It was found that the molecular segments for biosynthesizing lipids and other critical nutrients are abundant in humic substances in habitat soils, which may supply the energy and material sources for Thitarodes larval survival in the absence of sensitive plant roots, especially during the yearly cold winter (Li et al., 2019).

**Fermentation**

Many researchers are aiming for large-scale fermentation synthesis of *O. sinensis*, so that fungal strains may be readily separated from natural *O. sinensis* and generated in huge quantities using fermentation technology (Shashidhar et al., 2013). Due to difficulty in

producing a complete fruiting body of *O. sinensis*, researcher opt to producing mycelial biomass containing high bioactive components comparable to the wild *O. sinensis*. Mycelium is mass-produced artificially utilizing culture media, using two growing methods: liquid culture fermentation and solid substrate fermentation (Choda, 2017).

**Liquid culture**

Liquid culture or submerged fermentation applies by introducing *O. sinensis* into a tank of sterilized liquid medium containing all nutrients needed for rapid growth of mycelium (Holliday et al., 2004). Because of the simplicity with which the conditions may be controlled and modified with high mycelial production as proven for many fungi, this technique is the ideal technology for effective generation of desired bioactive chemicals by mycelial cultures (Shashidhar et al., 2013). However, because *O. sinensis* is acquired in liquid culture by straining, most of the essential extracellular components are removed during harvesting (Choda, 2017). This contributed to major loss of bioactive components since most of which are produced extracellularly in nature and only a small percentage can be found within mycelium (Holliday et al., 2004). Thus, to minimize loss, it can be suggested that the extracellular components of bioactive produced within liquid medium to be extracted and commercialized individually without the present of mycelium.

Previous studies conducted various parameters to assess the mycelial growth and biomass using submerged fermentation due to the commercial value of mycelia as fruiting body substitute as it is hard to cultivate artificially. Singh et al. (2014) evaluated nutritional requirements for in vitro culture of O. sinensis. It was disclosed that under optimized condition of potato dextrose broth (PDB; 30 g/L sucrose, 4 g/L beef extract, 10 mg/L folic acid, 1 mg/L calcium chloride, 500 mg/L zinc chloride), the yield of mycelium produced (12.08 g/L) was higher than the control (3.85 g/L). Meanwhile, Krupodorova & Barshteyn (2015) studied on the effect of amaranth flour as supplement for the growth of O. sinensis, and it was found that the mycelial biomass produced was 17.4 ±0.1 g/L higher than the previous report. Another researcher reported the effects of hypo- and hyperbaric pressures on O. sinensis mycelial growth. The result showed at –150 mmHg, the treated samples have the highest growth of mycelia (Gamage & Ohga, 2017).

On the other hand, Shashidhar et al. (2017) discovered in their research that the supplementation of coconut water in PDB enhanced the biomass yield of *O. sinensis*, especially mature coconut water (5.86 ± 0.33 g/L dw). Moreover, it also supported maximum production of adenosine, cordycepin, and polysaccharide (1.27 mg/g, 1.09 mg/g, and 6.41%). In addition

to mycelial biomass, exopolysaccharide (EPS) content was also investigated. Kim & Yun (2005) obtained maximum mycelial biomass and EPS (20.9 g/L and 4.1 g/L) using optimized culture conditions of 20 g/L sucrose, 25 g/L corn steep powder, 0.78 g/L $CaCl_2$, and 1.73 g/L $MgSO_4.7H_2O$. Another researcher studied the effect of ammonium feeding on mycelial biomass along with EPS and cordycepin. It was found that 10 mmol/L ammonium fed to the liquid culture (40 g/L glucose, 10 g/L yeast extract, 5 g/L peptone, 1 g/L $KH_2PO_4$ and 0.5 g/L $MgSO_4$) encouraged maximum biomass concentration, production of EPS (3.7 g/L), and cordycepin (117 ug/g) (Leung & Wu, 2007).

In addition, Wang et al. (2011) also studied on enhancing production of mycelial biomass and EPS via palmitic acid supplementation. The result revealed 1.0 g/L palmitic acid increase the concentration of mycelial biomass and EPS from 8.26 ± 0.34 to 11.10 ± 0.39 g/L and 353.1 ± 11.5 to 431.2 ± 13.8 mg/L, respectively. Besides biomass and EPS, other researchers also studied on the production of conidia, polysaccharide, glucosamine, and cordycepin. Ren & Yao (2013) evaluated nutritional requirements and physical stress conditions on conidial production. It was revealed that enriched potato dextrose agar (PDA; 5 % wheat bran, 0.5 % fish peptone, 0.1 % yeast extract, 2.5 % dried silkworm-pupa meal) showed increase germination, while physical stress of frozen-shock produced 7.5 times conidia higher than control. Hsieh et al. (2004) studied optimum medium condition for the production of polysaccharide and discovered maximum amount of 3.05 and 3.21 g/L in a shake flask and a 5-L jar fermenter, using optimized medium containing 6.17% sucrose, 0.53% corn steep powder, 0.5% $(NH_4)_2HPO_4$, and 0.15% $KH_2PO_4$ at pH 2.85.

Furthermore, another previous research has discovered the addition of 2 % citrus peel in optimized medium (1.5% rice bran, 0.5% molasses, 3% CSL, 0.1% $KH_2PO_4$, and 0.05% $MgSO_4$) improve EPS productivity as well as glucosamine content (48.9 mg/ml and 174.6 ug/ml, respectively) (Choi et al., 2010). Meanwhile, Kaushik et al. (2020) studied the effect of different growth supplements on the cordycepin production. Four types of growth supplements were utilized, including amino acids, nucleosides, plant growth hormones, and vitamins. The result depicted amino acid glycine at 1 g/L produced 434.97 ± 2.32 mg/L cordycepin, nucleoside hypoxanthine produced 466.48 ± 3.89 mg/L cordycepin, plant growth hormone NAA and IAA significantly increased the cordycepin content up to 227.61 ± 2.34 and 226.02 ± 1.69 mg/L, and vitamin B1 was found to be the most appropriate vitamin for potentiating the cordycepin production as cordycepin amount raised to 185.26 ± 2.35 mg/L on supplementation at 100 mg/L concentration in liquid medium. Table 1 summarizes the previous studies made on liquid culture/submerged fermentation.

**Table** 1. Summary of artificial cultivation via liquid culture fermentation of previous studies

| Carbon source | Nitrogen source | Culture conditions | | | | References |
|---|---|---|---|---|---|---|
| | | Temperature | Incubation | pH | Agitation | |
| Sucrose | Beef extract | 10 | | 5.5 | 200 | Singh et al. (2014) |
| Glucose, sucrose | Yeast extract | 26 | 14 days | 6.0 | | Krupodorova & Barshteyn (2015) |
| | | 25 | 16 | | | Gamage & Ohga (2017) |
| | | 28 | | 7.0 | | Shashidhar et al. (2017) |
| Sucrose | Corn steep powder | 20 | 4 | 4.0 | 150 | Kim & Yun (2005) |
| Glucose | Yeast extract, peptone | 25 | | | 150 | Leung & Wu (2007) |
| Glucose | Yeast extract, peptone | 27 | 7 | | 160 | Wang et al. (2011) |
| Sucrose | Corn steep powder, $(NH_4)_2HPO_4$ | 25 | 8 days | 5.0 | 300 | Hsieh et al. (2004) |
| Molasses, rice bran, | corn steep liquor | 25 | 5-6 days | 5.5 | 150 | Choi et al. (2010) |
| Sucrose | Corn steep powder | 20 | 10 days | 6.0 | 150 | Kaushik et al. (2020) |

**Solid culture**

Solid substrate fermentation, on the other hand, utilizes grains or mixed bag of cereals, such as rice, wheat, or rye, where the mycelium will grow. This technique is widely practiced in Japan and America (Holliday et al., 2004). The mycelium is gathered together with the remaining grain after some period of growth. The advantage of this method is that it is low-cost technique and able to produce mycelium with maximum bioactives recovery. However, mycelia produced has the drawback of having a higher content of grain matter than real *O. sinensis* ingredient (Shashidhar et al., 2013). In several cases, the residual grain content of the solid/substrate-grown mycelium evaluated exceeded 80%. The extracellular chemicals, however, are extracted with the substrate and mycelium, which is a benefit of this approach (Holliday et al., 2004). The application of solid culture commonly involves with the desire to produce fruiting bodies

instead of only mycelium formation. There are few studies conducted with the purpose of producing fruiting bodies using solid substrate fermentation.

A study was conducted by producing a hybrid called *C. sinensis* Alohaensis using snake venom as hybridization agent. It was proved to be a higher potency than the other artificially cultivated *C. sinensis* and it was easier cultivated on solid substrate (Holliday et al., 2004). In addition, Cao et al. (2015) performed an experiment on producing fruiting body in artificial medium containing 16 g/L of rice, 0.4 g/L of silkworm pupae powder, and 20 mL of nutrient solution (20 g/L glucose, 2 g/L $KH_2PO_4$, 1 g/L $MgSO_4$, 1 g/L ammonium citrate, 5 g/L peptone, and 20 mg/L vitamin B1. Three strains were investigated, and approximately 5 months, two out of three strains showed fruiting bodies formation. Besides, another study was executed to assess the utilization of ceramic beads as substrate alternative in producing fruiting body. It was found that the application of ceramic beads in the cultivation medium (350 g/L ceramic bead and 150 ml nutrient solution containing 50 g/L CMC-Na, 20 g/L potato powder, 20 g/L glucose, 2 g/L yeast extract, 2 g/L peptone, 0.5 g/L $MgSO_4 \cdot 7H_2O$, and 0.5 g/L $KH_2PO_4$) produced higher amount of fruiting bodies compared to control substrate (sawdust: rice bran: wheat bran, 8:1:1) (Huang & Ohga, 2017).

The mycelial growth of *O. sinensis* was also considered in solid state fermentation. Amin & Alam (2008) studied the growth of mycelia on different medium, and the result showed PDA has the fastest growing rate of 47 days with maximum mycelial thickness at pH 9 as well as minimum days of mycelial growth. In contrast, a study was carried out to investigate the effects of culture parameters on germination and yields of *O. sinensis* conidia. In their study, Mei et al. (2013) found medium containing 2.5% malt extract, 1.0% soluble starch and 0.9% agar was the best for conidial germination and optimal for harvesting conidial yield., which could be stimulated by the addition of soluble starch. Besides, the optimum condition for the production of polysaccharide by O. sinensis was explored by Wu et al. (2009). The parameters were conducted based on single-factor test, and it was revealed the optimum condition for production of polysaccharide were 20 % inoculation, fermentation at 26℃, 60 % water content of the medium (soybean and rice bran, 1:2), 60 % humidity, and 7-day fermentation time. Table 2 summarizes the previous studies made on solid culture fermentation.

**Table 2.** Summary of artificial cultivation via solid culture fermentation of previous studies

| Carbon source | Nitrogen source | Substrate | Culture conditions | | | | Ref. |
|---|---|---|---|---|---|---|---|
| | | | Temp. | Incubation | pH | Light | |
| Activated carbon | Light malt extract | | 20-22, 3 | 24 weeks | | | Holliday et al. (2004) |
| Glucose | Ammonium citrate, peptone | Rice, silkworm pupae powder | 9-13, 4, 13 | 190 days | | | Cao et al. (2015) |
| Sodium carboxy-methyl cellulose | Potato powder, yeast extract, peptone | Ceramic bead | 22, 15 | 114 days | | 500-lux | Huang & Ohga (2017) |
| Dextrose | Potato extract | | 25 | | 9.0 | | Amin & Alam (2008) |
| Soluble starch | Malt extract | | 14 | | 6.0 | | Mei et al. (2013) |
| | | Soybean & rice bran (1:2) | 26 | 7 days | | | Wu et al. (2009) |

## Quality Control of Artificially Cultivated *O. sinensis*

Natural *O. sinensis* is scarce and expensive, and it is accompanied by major environmental degradation, improper animal husbandry development, and indiscriminate long-term and over-excavation, all of which contribute to the shrinking in amount (Guo et al., 2021). With the advancement of modern biotechnology, the artificial production of *O. sinensis* has achieved unprecedented levels of complexity (Xiao et al., 2013). This circumstance has led to increased number of company marketing *O. sinensis* of artificial cultivated for the application in nutraceutical as well as pharmaceutical (Holliday et al., 2004; H. Hu et al., 2015). The popular artificial cultivation method adopted involved liquid culture fermentation and solid or substrate culture fermentation (Holliday et al., 2004). However, since there are no standardized quality

standards for fermented products of *O. sinensis*, the market has been flooded with products of varying quality (Chen et al., 2018).

As a result, quality monitoring of *O. sinensis* and its products is critical to assure their safety and efficacy (Kumar et al., 2013). In this case, active components are the focal focus and guarantee of quality control for raw materials. *O. sinensis* are well known for having various active components, including nucleosides, polysaccharides, sterols, amino acids, vitamins, flavonoids as well as trace elements (Chen et al., 2018; Shi et al., 2020). Some of these components has been used as markers for the purpose of quality control, such as nucleosides, polysaccharides, and sterols (Hu et al., 2015; Li et al., 2006). Among which, nucleosides are acknowledged as major active compounds in *O. sinensis* (Xiao et al., 2013; Yao et al., 2019), and the most reliable potency indicators (Holliday et al., 2004). The main nucleosides identified in *O. sinensis* includes adenine, adenosine, cordycepin, guanine, hypoxanthine, thymine, uracil, uridine, and 2-chloroadenosine (Guo et al., 2006).

Adenosine and cordycepin has been approved as significant markers of Cordyceps for quality control purpose (Li et al., 2001; Xiao et al., 2013). This is because these compounds are present within different species of Cordyceps but not in any other organism, which makes it unique to Cordyceps species (Holliday et al., 2004). In addition, besides being indicator of potency between natural *O. sinensis* and cultured one, these actives are also used to differentiate between *O. sinensis* and their counterfeit. Besides nucleosides, the comparison and characterization of polysaccharides from distinct Cordyceps species are critical to enhance the quality control of both wild and cultured Cordyceps (Wu et al., 2014). This is owing to the structural complicity of polysaccharides and rare materials (Wu et al., 2014). Sterols are also important bioactive components in *O. sinensis*, with ergosterol being the main compound. It has been found in Chinese Pharmacopoeia that the quality control of sterol involves identification and determination of ergosterol content (Jiaqian Zhao et al., 2020).

Various analytical methods have been implemented in quantifying the bioactives component within *O. sinensis* as a mean of quality check. In earlier days, redox titration was performed in determining mannitol content, but due to high interference from reductive components like glucose and fructose, the results obtained does not reflect a valid quantity of mannitol content (Li et al., 2006). This progressed to a new method of calorimetry, which is much simpler, specific, and rapid to determine mannitol and polysaccharides content (Li et al., 2006). Besides, for more accurate measurement and separation of mannitol, thin layer chromatography (TLC) was designed to minimize the effect of reductants interference. In fact, the application of TLC in the analysis of chemical has been used broadly since, due to its benefit of a wide range of

detection capabilities when it comes to assessing TCM, besides able to analyze several samples (Ma et al., 2004; Xiao et al., 2013).

The content of ergosterol and nucleosides (adenosine, guanine, and uridine) was also analyze using TLC in both wild and cultured Cordyceps (Li et al., 2006). Previous study used a TLC approach with two-step gradient elution and successfully separate eight nucleoside compounds usually found in *C. sinensis* samples (Ma et al., 2004). In addition, capillary electrophoresis (CE) has grown into a really flexible separation technology since the early 1980s, due to its advantage of high separation efficiency, analytical speed and cost, minimal solvent and sample consumption, and technique development in a short time (Ganzera, 2008; Xiao et al., 2013). However, one of the most significant advantages of CE is that there are variety of separation modes can be chosen from based on the substances and samples to be analyzed (Ganzera, 2008). Besides, CE does not require gradients and delivers greater separation with gradient elution because of its superior resolution power (Xiao et al., 2013).

The technique of CE is generally applied in the analysis of nucleosides in Cordyceps. Few research previously has conducted experiment using CE as method of separation. Using a calibrated electrophoresis strategy, three nucleosides of adenosine, guanosine, and uridine has been isolated and identified in less than 10 minutes (Li et al., 2001). Meanwhile, other researchers developed a simple CE technique for simultaneous measurement of six major nucleosides in natural and cultured *C. sinensis*, which includes adenine, uracil, adenosine, guanosine, uridine, and inosine by utilizing adenosine monophosphate as an internal standard upon the CE conditions has been optimized (Gong et al., 2004). However, the aqueous extract of Cordyceps is high in proteins, which can contaminate the capillary and compromise selectivity, precision, and accuracy (Li et al., 2006). This issue can be overcome by utilizing reflux extraction on the samples.

In addition, gas chromatography (GC) method is also adopted for the chemical analysis of Cordyceps due to its exceptional and flexible approach, which applied on volatile compounds (Li et al., 2006). Previous study analyzed the chemical constituents of the essential oil of *C. sinensis* via GS-MS, resulted in separation of 72 peaks and identification of 41 of them (Li et al., 2006). Besides, mannitol can also be analyzed by using GC. However, mannitol is a non-volatile carbohydrate compound having six hydroxyl groups. A group of researchers established a GC technique for mannitol quantification in *C. militaris* (Li et al., 2006). Because mannitol includes bifunctional hydroxyl groups, organic boronic acids are more helpful for derivatization than TMS or acetate. In addition, high performance liquid chromatography (HPLC) is a versatile analytical method for separation, measurement, and identification of a

broad range of constituents (Esteki et al., 2019). It is widely used for its exceptionally strong, simple-to-use, completely automatable, quick separation technology with excellent selectivity, sensitivity, and resolution that permits the separation of macromolecules with low volatility and thermal stability (Esslinger et al., 2014; Esteki et al., 2019).

HPLC may be equipped with different detectors, such as a UV detector, photodiode array detector (DAD), evaporative light scattering detector (ELSD), fluorescence detector (FD), and mass spectrometry (MS), to build a quantitative approach that is easy, stable, and reliable (Kamal & Karoui, 2015; Xiao et al., 2013). Besides, detectors of nuclear magnetic resonance (NMR), chemiluminescence (CL), and evaporative light scattering (ELS) has also been applied with HPLC (Esteki et al., 2019). HPLC, however, have significant drawbacks, such as the possibility for serious health consequences due to the enormous quantities of hazardous solvents utilised and the poor power for qualitative conclusions (Esteki et al., 2019). Few components of Cordyceps was determined using HPLC coupled with UV-vis detection, including adenosine, cordycepin, ergosterol, and other nucleosides (Li et al., 2006; J. Zhao et al., 2014). A group of researcher has separated and determined five nucleosides in Cordyceps using HPLC-UV method at 260 nm, including adenosine, cordycepin, 2'-deoxyadenosine, guanosine, and uridine (Ikeda et al., 2008).

Another researcher developed a new HPLC-DAD method and determined eleven nucleosides and bases simultaneously including adenosine, cordycepin, cytidine, cytosine, guanine, guanosine, inosine, thymidine, thymine, uridine, and uracil in natural and culture of *C. sinensis* at 260 nm (Yu et al., 2006). Besides, another group of researcher devised an HPLC-DAD technique for the quantitative detection of purine and pyrimidine bases such as adenine, cytosine, guanine, hypoxanthine, thymine, and uracil in wild and cultured Cordyceps (Fan et al., 2007). Apart from the mentioned approaches, fingerprint has gained popularity in the quality control of traditional Chinese medicine (TCM), due to practical and sensible approach in food and drug quality inspection (Esteki et al., 2019; Jiaqian Zhao et al., 2020). Moreover, this procedure has been approved by WHO, the FDA, as well as State Food and Drug Administration (SFDA) of China (Chen et al., 2018; Zhao et al., 2020). However, this is a qualitative analysis, which can only be applied to represent the general properties of herb contents and serve as a quality, consistency, and stability indication (Chen et al., 2018).

Chen et al. (2018) demonstrated in their study an efficient technique of fingerprint chromatography via HPLC in determining the quality of fermented products of *C. sinensis* in conjunction with SA, HCA, and QAMS method as it was a viable, practical, and efficient technique to better recognizing and fully evaluate the quality of fermented Cordyceps sinensis

products. The QAMS approach is simple and accurate for determining the five active components (uracil, uridine, adenine, guanosine, and adenosine) of fermented *C. sinensis* by RCF. Meanwhile, Shi et al. (2020) applied HPLC-fingerprinting together with NIRS and PLSR in assessing the quality control of total nucleosides in fermented Cordyceps powder during the production process. Zhao et al. (2020), on the other hand, demonstrated sterol fingerprinting using HPLC in combination with SA, PCA, HCA, and QAMS, which has strong specificity, good reproducibility, able to enhance the quality control system for fermented *C. sinensis*, as well as ensure the product's safety and stability.

**Commercialization of Artificially Cultivated *O. sinensis* for pharmaceutical applications**

The *O. sinensis* fungus internationally attracted attention when it was disclosed that some Chinese runners who set world records in 1993 used it as part of their training regimen (Buenz et al., 2005; Kharkwal, 2016). Traditionally, *O. sinensis* has been used in traditional Chinese medicine to treat asthma and other bronchial diseases, as well as to provide vitality and sexual potency (Elkhateeb & Daba, 2020). Due to advancement of modern science, numerous benefits of *O. sinensis* have been found. One of the most significant achievements is the discovery of cordycepin having a robust antibacterial effect against most bacterial species that have gained resistance to other regularly used antibiotics (Elkhateeb et al., 2019). Other main constituents of *O. sinensis* having therapeutic potentials including adenosines, amino acids, cyclofurans, polysaccharides, ergosterol, cordyglucans, cordycepic acid (mannitol), and cordycepin (Bhatt et al., 2018). Table 3 shows the health benefits of *O. sinensis* and their respective chemical components.

**Table 3.** The health benefits of *O. sinensis*

| Chemical components | Health benefits | References |
|---|---|---|
| Cordycepin | Antitumor, Antiviral, Anti-HI, Anti-malarial, Anti-leukemia | Yoshikawa et al. (2004), Wu et al. (2014), Wong et al. (2010), Nakamura et al. (2003), Elkhateeb et al. (2019), Kharkwal (2016) |
| Adenosine | Anti-inflammatory, Anti-arrhythmic, Enhance erythropoiesis | Nakav et al. (2008), Tsai et al. (2010), Bhatt et al. (2018) |
| Polysaccharides | Antidiabetic, Increase corticosterone level in plasma, Antioxidant, antiaging | Elkhateeb et al. (2019), Kiho et al. (1996), El-Hagrassi et al. (2020), Koh et al. (2003), Yamaguchi et al. |

| | | |
|---|---|---|
| | | (2000), Ji et al. (2009), Wu et al. (2014) |
| Nucleosides | Heart protection | Bhatt et al. (2018) |
| Superoxide dismutase (SOD) | Antisenescence | Zhu et al. (1998), Bhatt et al. (2018) |

Besides, the consumption of *O. sinensis* as a whole has been proven to be beneficial in overall health aspect. In a clinical trial, the administration of *O. sinensis* was reported to be effective in improving the function of kidney. In addition, it was also capable of enhancing the overall immunity of chronic renal failure patients (Elkhateeb et al., 2019). The liver functions for those suffering from post-hepatic cirrhosis was also improved by daily consumption of Cordyceps (Zhou et al., 2009). Another clinical trial on *O. sinensis* was conducted to verify on the improvement of fatigue through increased cold intolerance in elderly patients, and the results showed positive outcome (Wu et al., 2014; Elkhateeb et al., 2019). Furthermore, *O. sinensis* also has noticeable impacts on other organ systems, like central nervous system, respiratory system, and endocrine system (Elkhateeb & Daba, 2020). In addition, adenosine, deoxyadenosine, related adenosine type nucleotides, and nucleosides are all found in cordyceps extracts, which aid to stabilize heartbeat and rectify cardiac arrhythmias (Elkhateeb & Daba, 2020).

These health benefits provided by *O. sinensis*, either in natural or artificially cultivated form, has contributed to a strong and growing industry. It is estimated that *O. sinensis* production globally reached 85-185 tons per year (Winkler, 2009). Harvesting and selling non-cultivated Cordyceps can have a substantial influence on household income in the areas where it's harvested (Arora et al., 2013; Elkhateeb & Daba, 2020). Raw Cordyceps is sold for an average of US$ 1.34 per piece at the point of origin, which depends on the color of Cordyceps found as an indicator of quality as well as habitat (Pradhan et al., 2020). Cordyceps has sparked widespread interest and value, resulting in a wide range of commercial goods developed from these fungi all over the world (Elkhateeb & Daba, 2020). For example, Cordyceps CS-4 strain product was created with commercial value of supporting immune, respiratory, and cardiovascular system as well as promoting normal metabolic energy. Other products are commercialized as dietary supplements, focusing on supporting immune system, athletic performance, boosting libido, heart, and kidney function, as well as reducing aging effects.

## Conclusion

In summary, the artificial cultivation of *O. sinensis* plays vital role in many aspects as it could solve the deprivation of wild *O. sinensis* due to over-exploitation as well as lower the cultivation cost and time, especially via fermentation technique. The cultivation of mycelia instead of formation of fruiting bodies began to find a place among researchers and cultivators. This is due to the ability to manipulate the number of bioactive components produced within the mycelia. The advance in technology also has made it easier to determine and control the quality of the artificially cultivated *O. sinensis* so that it is on par or even better than the wild species. Additionally, there has been many products commercialized nowadays were produced from artificially cultivated instead of wild species. Although existing studies demonstrated the efficiency of *O. sinensis* cultivation methods and their quality control measure, additional studies might be needed to investigate further on producing artificially cultivated *O. sinensis* with better multiple bioactive components.

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