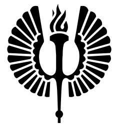


Liquid fermentation of *Ophiocordyceps sinensis* for bulk mycelium production

Master's Thesis in Technology
University of Turku
Department of Life Technologies
Biomolecular production
23.2.2026
Meeri Alppi

The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin Originality Check service.

Master's Thesis in Technology

Subject: Biotechnology
Author: Meeri Alppi
Title: Liquid fermentation of *Ophiocordyceps sinensis* for bulk mycelium production
Supervisors: M.Sc. Heikki Kiheri, M.Sc. Franz Bertenbreiter
Number of pages: 60 pages
Date: 23.2.2026

Ophiocordyceps sinensis, a parasitic caterpillar fungus, is a highly valued filamentous fungus containing several bioactive compounds. Those contribute to several bioactivities such as antioxidant, antitumor and anti-inflammation activities. These properties make *Ophiocordyceps sinensis* an area of interest in the nutraceutical industry.

Being an endangered species with high market demand, *in vitro* cultivation is studied to provide a renewable and stable source of the species. But its complex parasite-insect host relationship makes the artificial cultivation of the species challenging. Liquid fermentation produces mycelium, which contains bioactive compounds although in different concentrations than wild *Ophiocordyceps sinensis*.

The aim of this study is to utilize liquid fermentation to cultivate *Ophiocordyceps sinensis*, identify and optimize the key variables that affect growth to reach mycelium and bioactive compound levels that meet the industrial requirements. Different culture conditions (inoculation density, pH, duration, light and medium composition) are tested to find the ideal growth conditions. After cultivation, the mycelium is harvested by filtration and dried. Bioactive compounds are analyzed from dried mycelium with liquid chromatography and mass spectrometry. Dry weight and bioactive compound content of the mycelium are used to evaluate the differences between treatments.

The experiment showed that medium component concentration (glucose and yeast) had significant effect on the mycelium formation. The highest mycelium dry weight reached was 14.9±1.2 g/L after a 4-day culture, which is in the biomass range generally reported in literature (10-20 g/L). Significant effects were also detected from pH, duration and light experiments, but those did not result in as high biomass amounts as increased glucose and yeast concentrations did.

The dried mycelium contains bioactive compounds; such as adenosine, D-mannitol, ergosterol, ergothioneine and cordycepin; which are typical for *Ophiocordyceps sinensis*, but at lower concentrations than previously reported in literature. Only one tested treatment reached adenosine levels previously reported in the literature. Overall, the content of bioactive compounds was higher in treatments where biomass formation was lower, so compromises need to be made between the need for high biomass and bioactive compound formation. Based on the results cultivating the fungus in pH 4 instead of 6 would result in similar biomass formation but alter morphology, resulting in higher content of bioactive compounds in the mycelium.

Due to variation in growth and limited resources available (all the necessary variables could not be tested) optimization did not get as far as hoped for. A lot of testing and optimizing need to be done in the future to get the liquid fermentation process scaled into industrial scale.

Key words: bioprocess optimization; filamentous fungi; *in vitro* cultivation; liquid fermentation; nutraceutical; *Ophiocordyceps sinensis*.

Table of contents

Abbreviations 6

1 Introduction 7

1.1 Fungi as nutraceuticals/functional food 7

1.2 *Ophiocordyceps sinensis* 7

1.3* Nutritional value and bioactive compounds of *Ophiocordyceps sinensis 8

1.3.1 Nucleosides 9
1.3.2 Sterols 10
1.3.3 Amino acids 10
1.3.4 Polysaccharides 11
1.3.5 Other compounds 12

1.4 Artificial cultivation of *Ophiocordyceps sinensis* 12

1.4.1 *In vivo* cultivation 13
1.4.2 *In vitro* cultivation 13

1.5 Liquid fermentation strategy 14

1.5.1 Temperature 15
1.5.2 pH 15
1.5.3 Mixing 16
1.5.4 Oxygen transfer 16
1.5.5 Duration 16
1.5.6 Medium composition 17
1.5.7 Fungal strain 17
1.5.8 Challenges 18

1.6 Downstream processing 19

1.7 Commercial use of *Ophiocordyceps sinensis* 20

1.8 Quality control and identification 22

1.9 Aim of the study 23

2 Materials and methods 24

2.1 Fungal material 24

2.1.1 Making MYA plates 24
2.1.2 Re-plating/Transferring 25

2.2 Preparing seed cultures and liquid cultures 25

2.3 Optimization of liquid culture conditions and medium 27

2.3.1 Culture conditions 28
2.3.2 Medium 29

2.4 Harvesting mycelium, determining mycelium dry weight and wet weight 30

2.5 Bioactive compounds analysed with LC/MS 31

2.6 DNA extraction and sequencing 31

2.7 Statistical and data analysis 32

3 Results and discussion 33

3.1 Effect of culture conditions on mycelium 33

3.1.1 Duration 33
3.1.2 Inoculum density 34
3.1.3 Light 35
3.1.4 pH 36

3.2 Effect of medium composition on mycelium 38

3.3 Variation between replicates vs between experiments 41

3.4 Correlation between mycelium dry weight and wet weight 43

3.5 Bioactive compounds in the mycelium 44

3.6 DNA sequencing 48

3.7 General discussion and future perspectives 49

3.7.1 Limitations of the study 50
3.7.2 Future 52

4 Conclusion 53

Acknowledgements 54

References 55

Abbreviations

DoE	Design of experiments
DW	Dry weight
EPS	Extracellular polysaccharides
Glc	Glucose
IPS	Intracellular polysaccharides
ITS	Internal transcribed spacer
LC	Liquid Chromatography
MS	Mass Spectrometry
MYA	Malt Yeast Agar
NGS	Next generation sequencing
O. sinensis	*Ophiocordyceps sinensis*
PCR	Polymerase chain reaction
RO	Reverse osmosis
SD	Standard deviation
SOP	Standard operating procedure
Ww	Wet weight

1 Introduction

1.1 Fungi as nutraceuticals/functional food

"Higher fungi" is a term used to describe filamentous, fruiting body forming fungi (Fazenda et al. 2008). These kinds of fungi are known for containing many nutrients (carbohydrates, proteins, fats, fiber, amino acids, vitamins and minerals) and having benefits to human health (Azeem et al. 2020).

Edible mushrooms have been utilized for many millenniums as food, the earliest records going back to hieroglyphs in ancient Egypt (Azeem et al. 2020; El Sheikha and Hu 2018). On the other hand, fungi with medicinal properties have been used as treatments for different ailments (Azeem et al. 2020). For example, medicinal fungi has been used in traditional Chinese medicine as alternative medicine to treat different ailments for centuries (Azeem et al. 2020). Some fungi can be both edible and medicinal. Fungi can harbor many bioactive compounds such as polysaccharides, polyphenols, polyketides, proteins, nucleosides, terpenoids and steroids which give fungi pharmacological activities (antioxidant, antitumor, antimicrobial, among others) (Azeem et al. 2020).

Nutritional and medicinal properties have made fungi an area of interest, especially in the nutraceutical industry. Term nutraceutical refers to food products that also have some health benefits/pharmaceutical properties (Kityania et al. 2022). The market size for mushroom products (including nutraceuticals) is substantial, in 2024 the market value of cultivated mushrooms was 66.9 billion US dollars (The Business research company, 2025a). One particularly interesting fungus with both nutritional and medicinal properties is *Ophiocordyceps sinensis*.

1.2 *Ophiocordyceps sinensis*

Ophiocordyceps sinensis (O. sinensis) (Berk.) Sacc. (formerly known as C*ordyceps sinensis* (*C. sinensis*)) (Kiinanloisikka in Finnish) is a highly valued and well known fungus in Chinese traditional medicine (L.-Y. Wang et al. 2015; Zhu et al. 1998b). It is an ascomycetous higher fungus naturally found in high altitudes in Qinghai-Tibetan plateaus (Azeem et al., 2020; Yan & Wu, 2014; Zhu et al., 1998b). It is a parasitic caterpillar fungus that infects different caterpillar species, most commonly the moth larvae of *Hepilus spp.*, forming caterpillar-fruiting body complexes (Figure 1) (Azeem et al., 2020; Yan & Wu, 2014;

Zhu et al., 1998b). The fungus has an unusual lifecycle that begins during autumn when the fungus infects the caterpillar with fungal mycelia, which is released after the fungal spores have interacted with the chemicals present on the skin of the caterpillars (Zhu et al. 1998b). Fungal mycelia then consumes the caterpillar (underground) and by the spring/summer next year a fruiting body starts to form from the head of the dead caterpillar (Yan and Wu 2014; Zhu et al. 1998b).

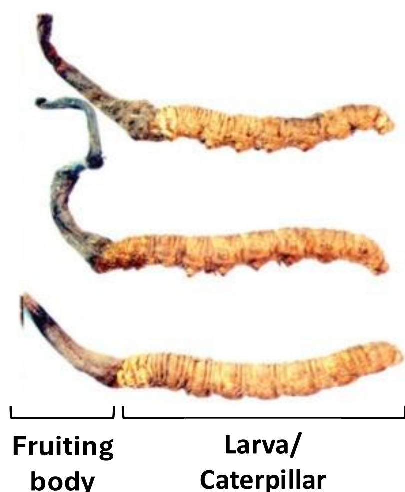


Figure 1 *Ophiocordyceps sinensis* caterpillar-fruiting body complex. Figure modified from Lo et al. 2013.

1.3 Nutritional value and bioactive compounds of *Ophiocordyceps sinensis*

O. sinensis is a highly valued and desired fungus due to its nutritional value and therapeutic effects (Ashraf et al. 2020; Krishna, Ulhas, et al. 2024; Yan et al. 2014; Yan and Wu 2014). In traditional Chinese medicine *O. sinensis* has been used to treat many different symptoms/illnesses like fatigue, hyperglycemia and heart, lung and liver diseases (Zhu et al. 1998b, 1998a). The bioactive compounds and bioactivities responsible for the interest around *O. sinensis* as a nutraceutical are illustrated in Figure 2. The price of *O. sinensis* material has increased over the years and for the best quality fungi price has been up to 100 000 $/kg, making *O. sinensis* one of the most expensive fungi in the world (Krishna, Balasubramanian, et al. 2024; Shrestha and Bawa 2013; Wei et al. 2021).

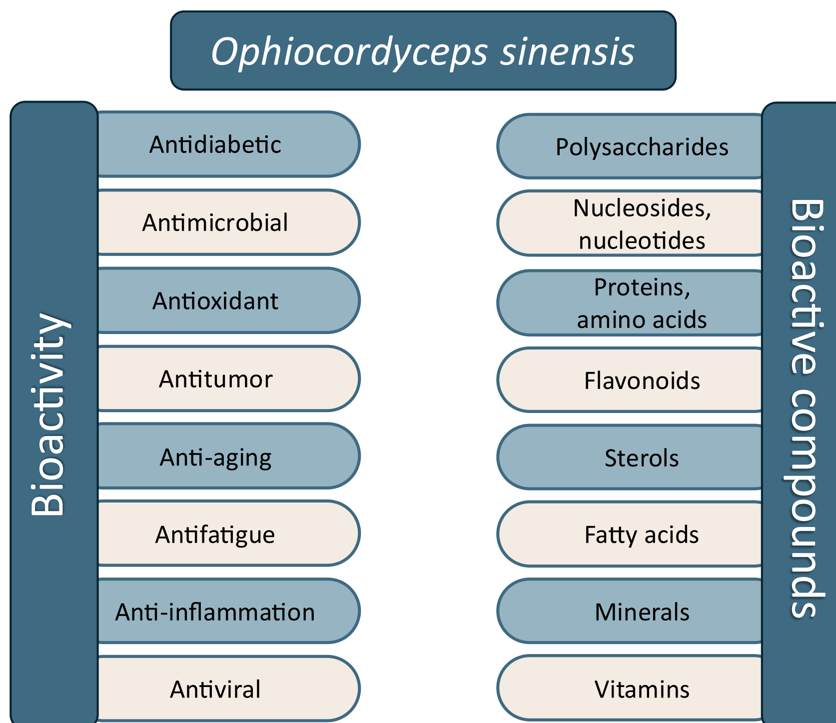


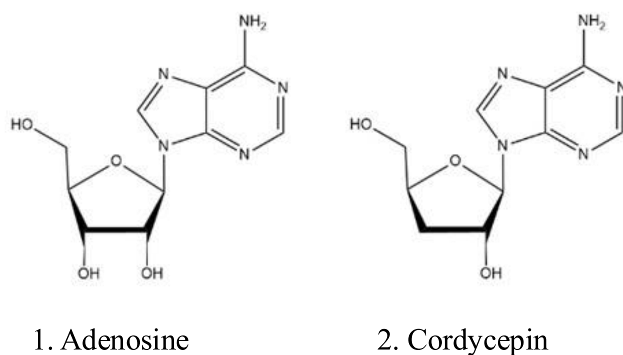
Figure 2. Bioactive compounds and bioactivities of Ophiocordyceps sinensis. Modified from Krishna, Ulhas, et al. 2024.

1.3.1 Nucleosides

Nucleosides are important bioactive compounds present in *O. sinensis*. In addition to their bioactive properties, they can be used as markers in quality control (Krishna, Ulhas, et al. 2024). For example adenosine has an important role as immunomodulator and quality control marker in addition of it being a building block for RNA/DNA synthesis and energy transfer (Adenosine triphosphate, ATP) (Chellapandi and Saranya 2024; Krishna, Ulhas, et al. 2024; Yi Liu et al. 2015).

Another important nucleoside in *O. sinensis* is cordycepin (3'-deoksyadenosine) which is an adenosine analog, missing one hydroxyl group compared to adenosine (Figure 3) (Krishna, Ulhas, et al. 2024). Because cordycepin is an adenosine analog, it is thought that its synthesis does not happen until DNA synthesis has ended (Krishna, Ulhas, et al. 2024). Cordycepin is mentioned to have various bioactivities like antitumor, anti-inflammatory, antimicrobial, antiviral and antidiabetic activity (Ashraf et al. 2020; Krishna, Ulhas, et al. 2024). These properties have made cordycepin and the cultivation of the fungus an area of interest (Krishna, Ulhas, et al. 2024). This compound was first found from *Cordyceps militaris* and there is debate on whether *O. sinensis* contains cordycepin or not (Krishna, Ulhas, et al. 2024). Some studies show that fruiting bodies of *O. sinensis* contain only small amounts of

cordycepin and fermented mycelium contains higher amounts (Yan and Wu 2014; Zhou et al. 2009). In contrast F. Q. Yang et al. 2010 say that cordycepin levels in natural *O. sinensis* are under quantification limits (0.04 µg/ml) and undetectable in cultured *O. sinensis* (<0.01 µg/ml).



1. Adenosine   2. Cordycepin

Figure 3. Chemical structure of adenosine (1) and cordycepin (2).

1.3.2 Sterols

Sterols are also significant bioactive compounds found in *O. sinensis.* One of the key sterols found is ergosterol (Figure 4). Ergosterol possesses antioxidant, antimicrobial and antiviral activities and is the provitamin form of vitamin D2 (Ashraf et al. 2020; Krishna, Ulhas, et al. 2024; Yi Liu et al. 2015).

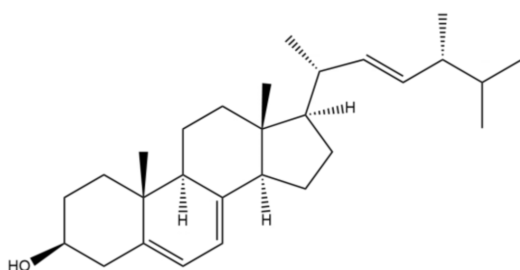


Figure 4. Chemical structure of ergosterol.

1.3.3 Amino acids

As mentioned, *O. sinensis* contains many amino acids and one in particular is ergothioneine which is a histidine derivative (Figure 5) found in foods such as mushrooms (R. W. S. Li et al. 2014). This compound has been found to harbor antioxidant activities and be a substrate to monocyte protecting cation transporter (Chen et al. 2012; R. W. S. Li et al. 2014).

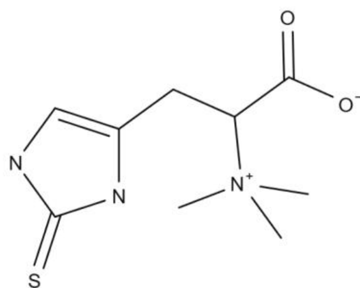


Figure 5. Chemical structure of ergothioneine.

1.3.4 Polysaccharides

Other major bioactive compound class found in *O. sinensis* is polysaccharides which can be divided into intracellular polysaccharides (IPS) and extracellular polysaccharides (EPS) based on where the polysaccharides are located in the fungal cells (Yan et al. 2014). Polysaccharides found in *O. sinensis* are responsible for various bioactivities like immunomodulatory, antitumor, antioxidant, hypoglycemic and anti-fatigue activities (Yan et al. 2014).

β-glucans are especially interesting bioactive polysaccharides that are present in *O. sinensis*. Generally β-glucans can be found from the cell walls of plants, fungi and yeast and also fungi are able synthesize some β-glucans (Cerletti et al. 2021; Rop et al. 2009). Their polymeric structure consists of glucose subunits that are linked together with β-1,3; β-1,4 or β-1,6, linkages, forming linear and branched structures **(**Figure 6**)**. β-glucans have shown several bioactivities; such as anti-inflammatory, immunomodulatory and antitumor activities (Cerletti et al. 2021; Rop et al. 2009). The presence of β-glucans in fungi are one of the main reasons for the use of fungi as nutraceuticals (Rop et al. 2009).

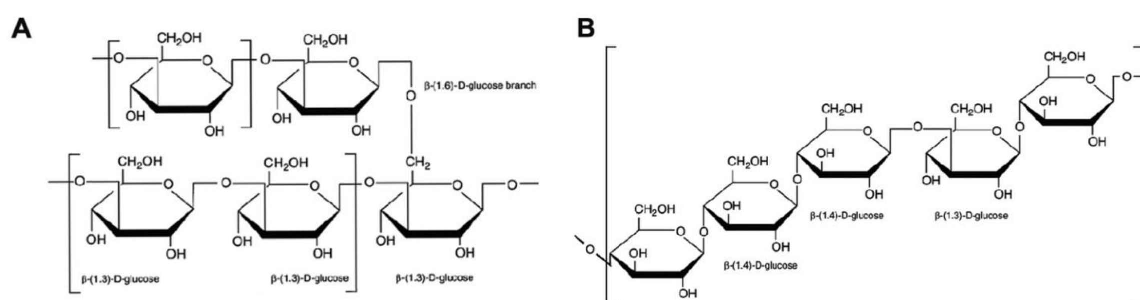


Figure 6. Structure of β-glucans containing β-1,3 and β-1,4 linkages. (A) branched structure, (B) linear structure. Figure modified from Cerletti et al. (2021), Licensed under CC BY 4.0.

1.3.5 Other compounds

As mentioned, *O.* sinensis contains many more bioactive compounds each having their own bioactivities (Figure 2). One compound worth mentioning is D-mannitol. D-Mannitol, also known as Cordycepic acid, is a polyol ($C_6H_{14}O_6$) that has been used in pharmaceutical and food industry due to its properties (Figure 7) (Zhou et al. 2009). It is a bioactive compound that gives *O. sinensis* diuretic and anti-free radical activities (Lin et al. 2016; Yi Liu et al. 2015).

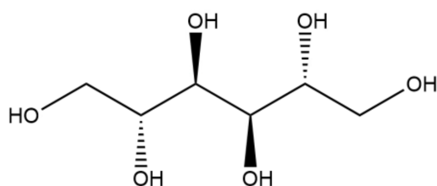


Figure 7. Chemical structure of D-Mannitol.

1.4 Artificial cultivation of *Ophiocordyceps sinensis*

Due to its relatively small natural habitat, climate change and high demand, overharvesting has led to declining *O. sinensis* populations and made it an endangered species (Shrestha and Bawa 2013; Wei et al. 2021; Yan and Wu 2014). Climate change is a critical threat to the natural habitat of the species. Based on Wei et al. (2021) *O. sinensis* prefers high elevation, high precipitation and low temperatures to grow and climate change is expected to lead to the warming and drying of Qinghai-Tibetan plateau. Meaning that the natural habitat of the species will shrink (Wei et al. 2021).

Artificial culture methods have been developed to provide more sustainable methods to satisfy the growing market demand and to conserve the species (Sharma et al. 2024). However, the artificial cultivation of *O. sinensis* is challenging because of its complex parasite-insect host relationship and specific environmental conditions needed for growth (Azeem et al. 2020; X. Li et al. 2019; Sharma et al. 2024; Yan and Wu 2014; Yao et al. 2024). While being more sustainable, the artificial culture methods additionally reduce the risk of contaminants associated with harvested fruiting bodies (Ko et al. 2017). For example, fruiting bodies harvested from the nature have been seen to sometimes cause heavy metal poisoning to the consumers due to the ability of fungi to accumulate heavy metals (such as lead, arsenic and copper) (Ko et al. 2017; Rop et al. 2009). These artificial culture methods can be divided into two categories: *in vivo* and *in vitro* cultivation (Figure 8).

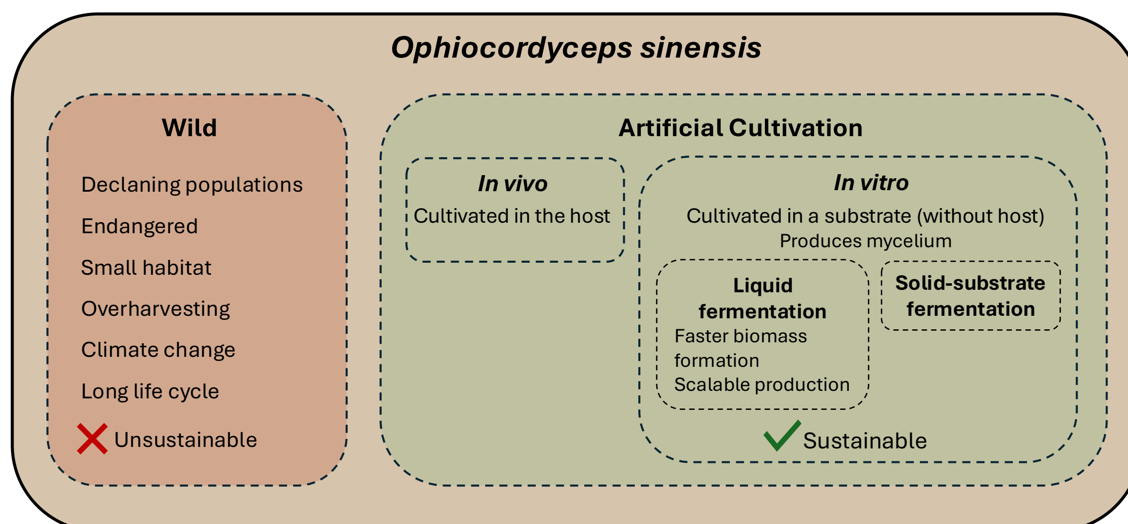


Figure 8. Summary of sources for *O. sinensis* material.

1.4.1 *In vivo* cultivation

In vivo cultivation utilizes the host of *O. sinensis* to mimic its natural environment. There have been attempts on developing culture methods for the host larvae so that *O. sinensis* fruiting bodies could be cultivated on a large scale artificially, but it is challenging due to the host's long life cycle (years) and low survival rate, low fungal invasion of the host and missing environmental cues (Ko et al. 2017; X. Li et al. 2019; Sharma et al. 2024). There have been successful attempts at growing the *O. sinensis* fruiting bodies, but it is still challenging, time-consuming and costly cultivation method that can't reach the market demand (X. Li et al. 2019; Sharma et al. 2024).

1.4.2 *In vitro* cultivation

Another artificial culturing method is *in vitro* cultivation where the fungus is cultured in a nutrient rich medium in controlled conditions, without the presence of the insect host, to produce fungal mycelium (Ko et al. 2017; X. Li et al. 2019; Sharma et al. 2024). *In vitro* cultivation provides a renewable and stable source of *O. sinensis* material, by producing mycelium, compared to harvesting wild *O. sinensis* (Yan and Wu 2014). The cultivated mycelium has been seen to have similar chemical composition as natural *O. sinensis* fruiting-bodies (Ko et al. 2017; Yan and Wu 2014). *In vitro* cultivation can be divided into solid state/substrate fermentation and liquid/submerged fermentation (Sharma et al. 2024).

Solid state/substrate fermentation is widely used for the cultivation of mushrooms/fungi (fruiting bodies) with grain as the substrate and it can be used for the cultivation of *O. sinensis* mycelium (Sharma et al. 2024). It is a cost-effective method to produce mycelium and to recover bioactive compounds (Sharma et al. 2024). Downside in this method is that the resulting mycelium has high residual grain concentration (Sharma et al. 2024). Because of this, solid-substrate fermentation is better suited for cultivating fungi that can produce fruiting bodies than to producing *O. sinensis* mycelium (Sharma et al. 2024).

Liquid/submerged fermentation on the other hand is used to cultivate *O. sinensis* in a liquid medium and it allows specific controlling of the cultivation conditions and separation of the mycelium from the culture medium (Sharma et al. 2024). By optimizing the culture conditions, it is possible to affect the formation of mycelium and bioactive compounds (Sharma et al. 2024; Yan et al. 2014; Yan and Wu 2014). One of the possible downsides in the liquid fermentation strategy is that during the harvesting step extracellular bioactive compounds are lost with the culture medium (Sharma et al. 2024).

Compared to solid-substrate fermentation, liquid cultivation provides a faster and more easily scalable method to produce mycelium (Dong and Yao 2005; Fazenda et al. 2008). And from all the artificial culture strategies it is currently the most promising approach to large-scale production. Due to these reasons, liquid fermentation strategy is focused on in more depth.

1.5 Liquid fermentation strategy

Liquid fermentation can be optimized by changing the cultivation conditions and medium composition in order to get the desired/maximum growth and manipulate the production of wanted bioactive compounds (Yan and Wu 2014). To reach the desired growth conditions bioreactors are utilized. A Bioreactor provides a closed, controllable environment where biological reactions take place which can be utilized for the large-scale production of fungal mycelia (El-Enshasy 2007; Yan and Wu 2014). Bottle cultures can be utilized in small scale experiments and before transitioning into bioreactor experiments. Both methods are widely utilized in *O. sinensis* studies but the parameters that can be tested and their effects can vary between the methods used. The main factors that affect the performance of the cells and need to be optimized in a liquid culture are presented in Figure 9 (El-Enshasy 2007; Fazenda et al. 2008). Some of the factors are discussed more in depth below.

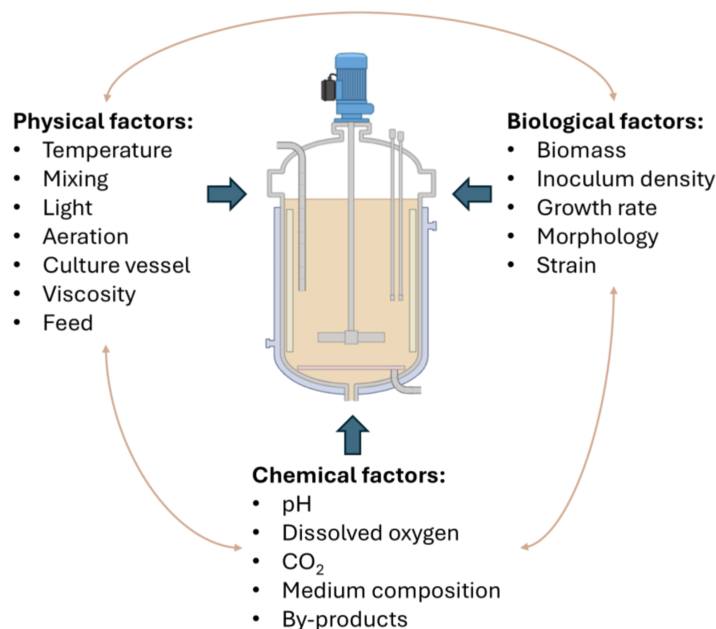


Figure 9. The main factors affecting liquid fermentation process. Figure created with BioRender. Modified from El-Enshasy 2007; Fazenda et al. 2008.

1.5.1 Temperature

Temperature is one of the most important factors to consider. In nature, *O. sinensis* requires low temperatures to grow (Wei et al. 2021). The low temperature requirement has also been seen in liquid cultures. *O. sinensis* grows in temperatures between + 4 to 21 °C, stopping altogether in temperatures over 25 °C (X. Li et al. 2019; Lo et al. 2013). L.-Y. Wang et al. (2015) and Ko et al. (2017) tested culturing *O. sinensis* liquid cultures in different temperatures (from 12 to 25 °C) to find the optimal liquid fermentation temperature and saw maximum growth in temperatures ranging from 15 to 18 °C.

1.5.2 pH

pH is an important factor that can affect cell membrane functions, morphology and structure of the cells, solubility of nutrients, substrate uptake and formation of biomass and bioactive compounds (Fazenda et al. 2008; L.-Y. Wang et al. 2015). L.-Y. Wang et al. (2015) observed the effects of pH (4-8) on the mycelium growth of *O. sinensis* and saw that initial pH of 6 reached the maximum mycelium growth. *O. sinensis* needs slightly acidic conditions for growth in liquid cultivation. In a cell culture where pH is not controlled, pH can change when fungal cells use nutrients and excrete waste-products which might affect the mycelium formation and biosynthesis of bioactive compounds (Fazenda et al. 2008).

1.5.3 Mixing

Proper mixing is vital for mycelium formation in liquid cultures. It keeps nutrient concentrations even in the culture medium and affects oxygen and heat transfer (El-Enshasy 2007; Fazenda et al. 2008; L.-Y. Wang et al. 2015; Yan and Wu 2014). There are different types of mixing equipment available. Depending on the culture vessel the mixing can be from the inside of the culture with impellers (bioreactor) or from the outside by placing the cultures onto a shaker (bottle culture) (Fazenda et al. 2008).

Mixing also causes shear forces which can damage cells, affects the morphology of the fungal cells, affect the growth rate and formation of bioactive compounds (Fazenda et al. 2008). L.-Y. Wang et al. (2015) tested how different rotation speeds on a rotary shaker (100 rpm, 150 rpm and 200 rpm) affect the mycelium growth. They saw that mycelium yield increased when speed increased from 100 rpm to 150 rpm but decreased drastically when the speed was increased to 200 rpm, which could be due to shear stress (L.-Y. Wang et al. 2015). Due to the benefits of higher mixing speed on oxygen transfer and nutrient concentration and the negative effects increased shear stress causes, balanced mixing speed is needed for best results.

1.5.4 Oxygen transfer

O. sinensis is an aerobic fungus, therefore adequate oxygen transfer is necessary for mycelium growth in liquid fermentation (Yan and Wu 2014). Oxygen supply and the dissolved oxygen (DO) levels have an effect to the growth rate and biosynthesis of bioactive products (Yan and Wu 2014). Oxygen transfer rate is directly linked to the mixing, with higher mixing speeds oxygen dissolves better and is more equally distributed in the culture medium (Fazenda et al. 2008; L.-Y. Wang et al. 2015). Cultures can rely on filtered air that is transferred to the medium by mixing or cultures can be supplemented with air or pure oxygen to improve the dissolving of oxygen into the culture medium (Fazenda et al. 2008).

1.5.5 Duration

The duration of the cultivation must also be considered because it affects the biomass yield, chemical composition and medicinal property of the biomass (Yan and Wu 2014). In *O. sinensis* liquid cultivations the general cultivation time to reach maximum biomass concentration has been seen to be 4 to 8 days, but also longer durations such as 40 days have

been reported (Dong and Yao 2005; Ko et al. 2017; R. Li et al. 2010; Yan et al. 2014; Yan and Wu 2014).

Inoculum method can significantly affect the needed duration of the culture. If cultures are inoculated from a plate culture, the fungus needs time to adapt (lag-phase) to the liquid culture whereas seed culture allows the adaptation to the liquid medium and when that is used as inoculum it can reduce the possible lag phase prior to reaching exponential growth stage (Fazenda et al. 2008).

1.5.6 Medium composition

The composition of the liquid medium is an important factor for growth because it provides the necessary components for the biomass and bioactive compound formation (Fazenda et al. 2008). Different medium compounds promote different effects and affect what compounds are formed (Fazenda et al. 2008).

Carbohydrates and their high concentrations are associated with higher biomass yields (Fazenda et al. 2008). Many different carbon sources have been used in culture mediums according to literature related to *O. sinensis*. The most utilized carbon sources seem to be glucose and sucrose and based on L.-Y. Wang et al. (2015) those generate most biomass and bioactive polysaccharides, but the choice of carbon source can affect the chemical composition of the mycelium.

Nitrogen source is also necessary for growth and bioactive compound formation and can have effects on the morphology of the fungus (Fazenda et al. 2008; L.-Y. Wang et al. 2015). Based on L.-Y. Wang et al. (2015) and Yan & Wu (2014) organic nitrogen sources such as yeast extract and peptone promote more mycelial growth and bioactive polysaccharide formation in *O. sinensis* than inorganic nitrogen sources like nitrates.

Supplementing the medium by adding minerals into it can be beneficial for the biosynthesis of bioactive compound such as polysaccharides and can promote the mycelium growth (Fazenda et al. 2008; L.-Y. Wang et al. 2015).

1.5.7 Fungal strain

Suitable fungal strains are needed for liquid cultivation that have efficient growth which can be scaled from laboratory scale up to industrial scale. But a problem was encountered while

doing research on the topic. In scientific literature there is confusion about what really is considered as *O. sinensis* material. Lot of sources mention that many “strains” have been isolated from the wild *O. sinensis*, which have then been used to cultivate mycelium in solid or submerged/liquid fermentation (Yan et al. 2014; Yan and Wu 2014; Yao et al. 2024; Zhu et al. 1998b). And some of the “strains” have been utilized in large scale mycelium production which has then been used in commercial products (Yan and Wu 2014; Yao et al. 2024). The “strains” have been thought to be *O. sinensis* based on morphology and chemical composition, and because they have been isolated from fruiting bodies of *O. sinensis* (Ko et al. 2017). After DNA sequencing techniques have become more utilized, it has been realized that many of these “strains” are completely different species and have been fungal contaminants present on the fruiting bodies of *O. sinensis* (for example sequencing has identified CS-4 as *Paecilomyces hepiali*) (Ko et al. 2017). This has led to a problem where incorrect strains are being called as *O. sinensis* in the scientific literature and global market (Ko et al. 2017). To reduce the uncertainty around the legitimacy of the fungal material used, DNA identification of the material should be done before further studies are done or products are introduced to the consumer market.

1.5.8 Challenges

Liquid cultivation of *O. sinensis* has also some challenges. Mixing and oxygen transfer might become challenging during cultivation if the culture medium’s viscosity increases and when biomass amount increases (Yan and Wu 2014). Additionally, fungi are prone to contamination which can then spoil entire production batches (Fazenda et al. 2008). In general, the slow growth rate of macrofungi like *O. sinensis* can make the optimizing and controlling of the cultivation process more challenging compared to bacteria and other industrial host organisms (Fazenda et al. 2008).

In addition to the challenges mentioned above, the culture condition requirements can be different for reaching maximum mycelium growth and for biosynthesis of bioactive compounds (Fazenda et al. 2008; Yan and Wu 2014). This is because biomass formation happens during primary metabolism (essential for growth) and the biosynthesis of bioactive compounds happens during secondary metabolism (not essential for growth but have survival functions) which usually happens after primary metabolism or under suboptimal growth conditions (Demain 1986; Nielsen and Nielsen 2017). For example, during active growing stage the synthesis of secondary metabolites is commonly suppressed (Demain 1986).

Therefore, sometimes compromises need to be made between which outcome is more desired, high yield or content of bioactive compounds. Other possibility is to test if altering the cultivation conditions to first support the generation of biomass and then changing them to support bioactive compound formation would result in high yield and bioactive content.

1.6 Downstream processing

As important as the optimization of liquid culture conditions is for the mycelium formation, the downstream processing also needs to be considered to get the fermented mycelium into a commercial product (Figure 10).

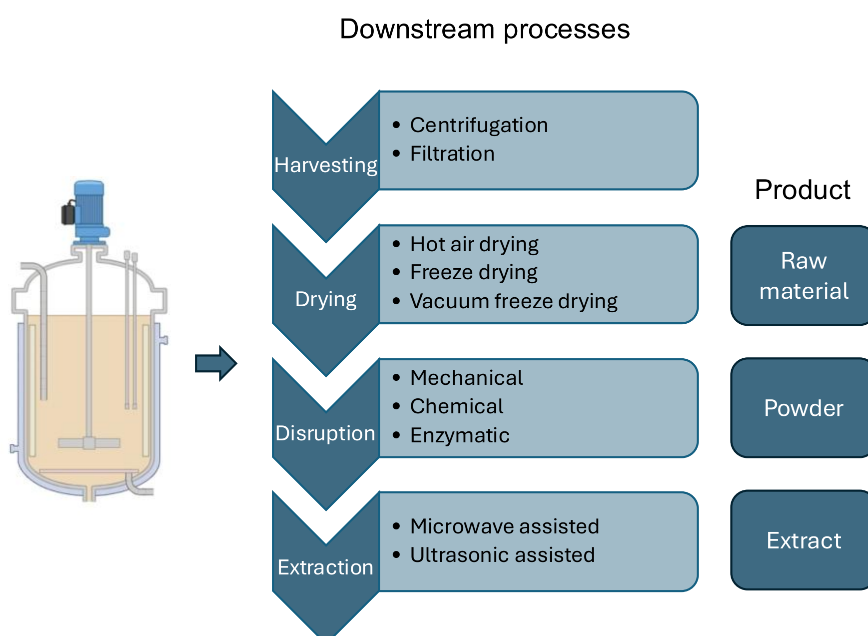


Figure 10. Illustration of different stages of downstream processing mycelium can go through after fermentation to become final nutraceutical product. Figure modified from Luo and Li 2022.

After the liquid culturing, the mycelium needs to be separated from the culture medium by harvesting. Several different harvesting methods can be utilized depending on the scale of fermentation. Typically, filtration or centrifugation is used. In small-scale fermentations laboratory centrifuges might be usable but when transferred to a larger scale more powerful equipment is needed to process increased volumes. Industrial scale centrifugation equipment can operate by continuously feeding culture medium into the equipment to separate solids from liquids by creating a centrifugal force after which the separated phases can be continuously collected through separate outlets (Dryden et al. 2021; Jungbauer 2013). Filtration on the other hand utilizes a porous material (filter medium, membrane or cloth) that allows materials to pass through based on their size (Doran 1995; Dryden et al. 2021). In a

small-scale, different porous materials can be utilized but on a larger scale the filtration requires a filtration equipment that uses vacuum or positive pressure to help the liquid flow through the filter (Doran 1995). Problem with traditional filtering is that during filtering process the solid material that doesn't pass through forms a filter cake on top of the filter, which generated resistant to the filtration and can clog the filter (Doran 1995). This can be overcome with rotary-drum vacuum filters which uses a rotating drum of which the formed filter cake is removed constantly (Doran 1995). Another possible filtration technique is to utilize tangential flow filtration on which the flow is tangential to the membrane, so there is no filter cake formation on the surface of the filter (Doran 1995). It is mentioned that the filtering of fungal mycelium is not as challenging as yeast and bacteria because mycelium is larger and has higher porosity (Doran 1995).

After mycelium is harvested it is dried to preserve the material (Luo and Li 2022). Without drying the fungal material would undergo spoilage (changes in texture, bioactive composition and have microbial growth) due to moisture and enzymatic activity still present (Luo and Li 2022). Several drying methods are available, for example hot air drying, vacuum drying and freeze drying, but the method used has an effect on the quality of the material which needs to be considered when deciding on which drying method is going to be used (Luo and Li 2022). With *O. sinensis* mycelium hot air drying and vacuum freeze drying have been utilized to obtain dry mycelium (Dong and Yao 2005; Ko et al. 2017; L.-Y. Wang et al. 2015). The dried *O. sinensis* can be stored and used as a raw material for nutraceuticals (Luo and Li 2022).

The downstream process can be continued by disrupting the dried fungal material to get fine powder in which bioactive compounds are more easily available (Luo and Li 2022). Mechanical, chemical and enzymatic disruption techniques can be utilized to disrupt the structure of which the mechanical techniques are the most cost-effective (Luo and Li 2022). The resulting fine fungal powder could be used as nutraceutical as it is or formulated further into tablets or capsules. In some cases, the processing of *O. sinensis* is continued from the mycelium powder by making fungal extract by utilizing for example microwave or ultrasonic assisted extraction, that make the product more concentrated and/or more bioavailable (Kityania et al. 2022; Luo and Li 2022; Sharma et al. 2024; Strong et al. 2022).

1.7 Commercial use of *Ophiocordyceps sinensis*

O. sinensis has a lot of commercial potential due to its therapeutic effects and nutritional value. There are several nutraceutical products made from *O. sinensis* currently on the global

market, product formulation ranging from powders to capsules and extracts (Sharma et al. 2024). Some commercial products are made from the highly valued and overharvested fruiting bodies, and some are made from fermented mycelium (Sharma et al. 2024). The global nutraceutical market (value 458.55 billion US$ in 2024, Fortune business insights (2025)) has grown along consumers increased health awareness, including the commercial use of *O. sinensis* (Ashraf et al. 2020). The global market size of *O. sinensis* was 1.21 billion US$ in 2024 (Natural and cultivated *O. sinensis*) (The Business research company, 2025b). Products made from *O. sinensis* are generally marketed as a highly valued fungi that has been used in traditional Chinese medicine due to its health promoting properties. In the European Union (EU) the use of *O. sinensis* (its mycelium and fruiting body) is allowed in food supplements (and it is not considered a novel food because it has been used in food supplements before May 1997) (European Commission).

Identifying the real origin and labeling of Ophiocordyceps products is problematic. The previously mentioned problem of completely different species being regarded as *O. sinensis* can also be seen on the nutraceutical market. For example, Cs-4 strain isolated from *O. sinensis* in 1982, was one of the first isolated “strains” suitable for industrial fermentation and after that several products were commercialized. There are still currently products on the market labeled as *(Ophio)cordyceps sinensis* made from Cs-4 even though it is currently known as its own species *Paecilomyces hepiali* (Ko et al. 2017; Zhu et al. 1998b). Furthermore, in mycelium products’ descriptions, the strain utilized is not always mentioned so it can’t be known if the products are in the end made from *O. sinensis* material or from other closely related species. In some cases, the materials’ country of origin is hard to identify. From some of the products on the market, it is hard to identify based on product descriptions whether the product is made from fruiting bodies or mycelium. In addition, the labeling regarding the bioactive compounds present in the commercial product has been seen to be insufficient (Sharma et al. 2024).

Because it is a highly valuable fungus with limited supply, there have been adulterations in the market. Adulterations (aim to increase profit), where other fungi or even metal has been added to the product, have been found on the market (Ko et al. 2017; Yang Liu et al. 2017). Proper quality control is needed to ensure consumers’ safety, origin of fungal material and composition of the product.

1.8 Quality control and identification

Several techniques have been utilized in quality control of *O. sinensis*. One approach rely on the analysis of chemical compounds of the fungal material to distinguish natural and cultured *O. sinensis* and *Cordyceps militaris* from each other (S. P. Li et al. 2006). For example, nucleosides such as adenosine and cordycepin, ergosterol and D-Mannitol can be used as markers in quality control (S. P. Li et al. 2006; Sen et al. 2023). Liquid chromatography (LC) combined with mass spectrometry (MS) is widely utilized for the analysis of chemical compounds from *O.* sinensis material (S. P. Li et al. 2006; Sen et al. 2023; F. Q. Yang et al. 2010). Problem is that the content of bioactive compounds can vary even between the same material type. For example, in natural *O. sinensis* from different geographical regions or in cultured mycelium made with different strains and culture conditions change the chemical composition (S. P. Li et al. 2006; F. Q. Yang et al. 2010). For this reason, it is more accurate to look at the chemical profile of the sample and not solely the content of one specific marker in the fungal samples (S. P. Li et al. 2006). In order to the quality of the fungal material/products to be constant, an standardized international analytical method would be needed to assure that same methods are used and the evaluation criteria is same globally (Sen et al. 2023).

DNA sequencing is also utilized in quality control to authenticate the fungal material (Lam et al. 2015). Different sequencing approaches are available such as full genome sequencing and amplicon sequencing/DNA barcoding. Amplicon sequencing allows the identification to be made based on a specific DNA region that is unique to each species. One such usable DNA region is nuclear ribosomal internal transcribed spacer (ITS) region (Figure 11) which has been used as a DNA barcoding marker for identification of fungi (Bellemain et al. 2010; Lam et al. 2015). What makes it ideal is that ITS sequencing is usable even in low DNA concentration samples due to the fact that a single cell can have several copies of the ITS region (Bellemain et al. 2010).

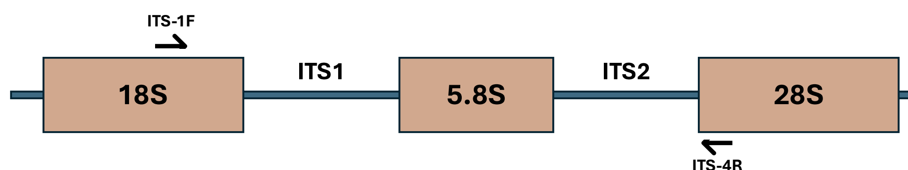


Figure 11. Internal transcribed spacer (ITS) region with ITS-1F and ITS-4R primers that can be used to amplify the whole fungal ITS region.

By combining sequencing to identify the fungal strain and the chemical profiling of the end products the quality of the *O. sinensis* products could be better ensured.

1.9 Aim of the study

The aim of the study is to get *O. sinensis* to grow in the laboratory environment on a small-scale with liquid fermentation strategy. The aim is to optimize the culture conditions to find the best conditions for biomass and bioactive compound formation and reach levels that are comparable to previous studies. The key variables affecting the growth of the fungi need to be identified and tested. The different variables/parameters that can be tested are medium compositions, inoculum density, temperature, pH, light, mixing and duration of cultivation.

If the laboratory scale tests perform well in a short time the aim is also to test the process on a larger scale in a bioreactor. But if there is no time for bioreactor experiments, then the aim is to get enough data from the small-scale experiments that can be used in the future to plan industrial production.

2 Materials and methods

The setup for the experiment is summarized in Figure 12.

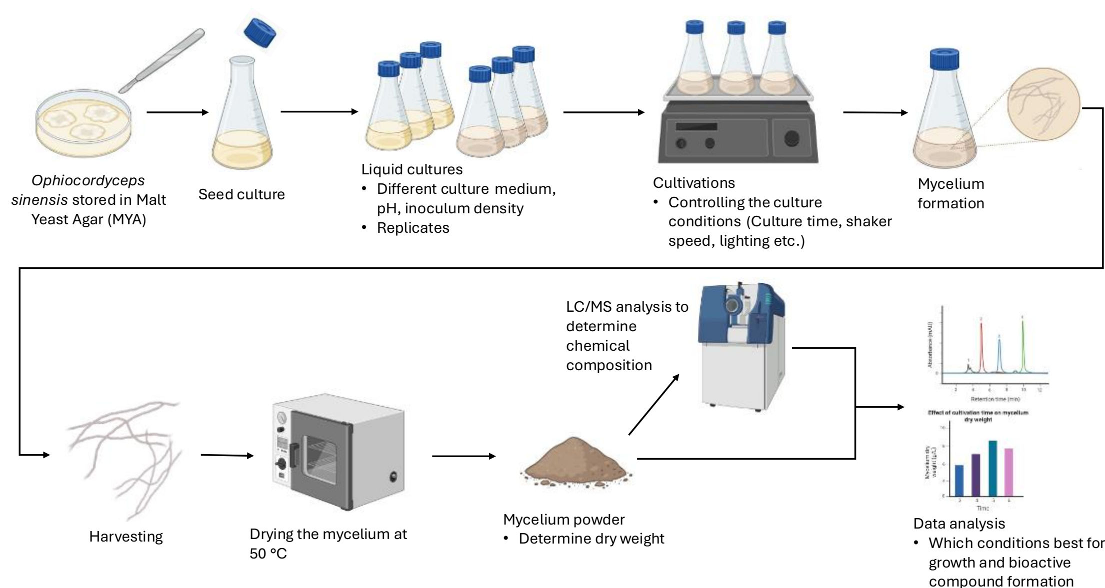


Figure 12. Illustrated summary of the methods used in this study. Figure created in BioRender.

2.1 Fungal material

O. sinensis material was found in Kääpä Biotech's laboratory. The fungal material was stored in Malt Yeast Agar (MYA) plates. The *O. sinensis* mycelium cultivated in this study was sent for DNA sequencing to identify and confirm the used fungal strain.

2.1.1 Making MYA plates

The plates were made by following Kääpä Biotech's Standard Operating Procedure (SOP). Organic agar (Biovegan), malt extract powder (DO IT Organic) and yeast flakes (DO IT organic) were weighed and dissolved to reverse osmosis (RO) water (Table 1). Agar medium was autoclaved at 121 °C for 30 minutes. Agar (cooled down to 80 °C) was poured into the plates (AHN myPlate, Sterile Petri dish. 90 mm) inside a laminar flow hood and the lids were left partially open for 15 minutes to allow rapid cool down. After cooling, the lids were closed. Plates were inoculated or sealed with parafilm M (Bemis) and stored in the fridge at +4 °C.

Table 1. Composition of Malt Yeast Agar.

Component	Amount (g/L)
Organic agar	23
Organic malt extract	22
Organic yeast flakes	2

2.1.2 Re-plating/Transferring

Fungal material was transferred into a fresh plate with a sterile scalpel by cutting a small square of agar covered with mycelium and transferring it into the middle of the fresh MYA plate (Figure 13). All plates were sealed with parafilm M and moved to the grow room (20-22 °C). Original plates were resealed with parafilm and stored at +4 °C. Plates were grown at room temperature until the mycelium reached edges of the plate (approximately 5-6 weeks) and then transferred into the fridge (+4 °C). Fungal material was transferred to fresh plates after 2 months.

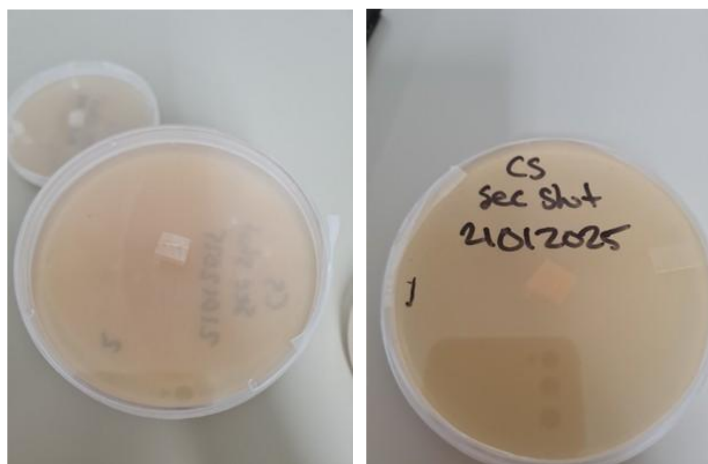


Figure 13. Piece of mycelium covered with agar transferred into fresh MYA plate.

2.2 Preparing seed cultures and liquid cultures

Experiments were performed by utilizing Kääpä Biotech’s standard liquid culture medium containing malt extract, yeast flakes, corn glucose (DO IT Organic) and gypsum flakes (CaSO4) dissolved in RO water (Table 2). This medium was used for both seed cultures and liquid cultures (control medium).

Table 2. Composition of liquid culture medium.

Component	Amount (g/L)
Organic malt extract	15
Organic yeast flakes	3
Corn glucose	28.6
Gypsum	2.9

In this study the fungus was cultured in culture cups, shown in Figure 14, which were 520 ml cups with lids containing a depth-filtration system ($SacO_2$, Microbox O95/114+OD95). The cups were filled with 250 ml (×(100 %−inoculum density %)) of culture medium and autoclaved at 121 °C for 30 minutes. Culture medium was cooled down overnight inside a laminar flow hood before inoculation. Volume of the medium was kept at ½ of the culture cup volume to allow adequate mixing and gas exchange.

Figure 14. 520 ml culture cups used in this study.

Seed cultures were inoculated inside a laminar flow hood with *Ophiocordyceps sinensis* 'Sec shot' grown on MYA plates by cutting three squares of agar (from the edges of growth) with sterile scalpel and dropping them into the culture cups. Seed cultures were grown at room temperature (20-22 °C) on laboratory shakers (Steinberg systems, SBS-LVS-100) at 140 rpm[1] for 4 days. Seed cultures were then stored in a fridge (+4 °C) until used as inoculum for experimental cultures

The experimental liquid cultures were inoculated with seed culture to reduce possible lag phase (Fazenda et al. 2008). Seed cultures[2] were homogenized with laboratory mixer (Steinberg systems, SBS-ER-3000) by changing the mixing speed from low to high, repeating

[1] Shaker speed chosen based on L.-Y. Wang et al. (2015) and Yan and Wu (2014)
[2] Chosen based on visual appearance (most dense looking cultures).

it a few times. Cultures were inoculated by pipetting homogenized seed culture into the cups with 5 ml pipette[3] (Sartorius) using inoculum density of 4 % based on L.-Y. Wang et al. (2015) testing inoculum densities of 3 and 5 %. The culture cups were placed on a laboratory shaker at 140 rpms and generally cultivated for 4 days (Figure 15). Storage boxes (Smart storage, 34x25x16 cm) were used to keep the cups from falling out of the shakers.

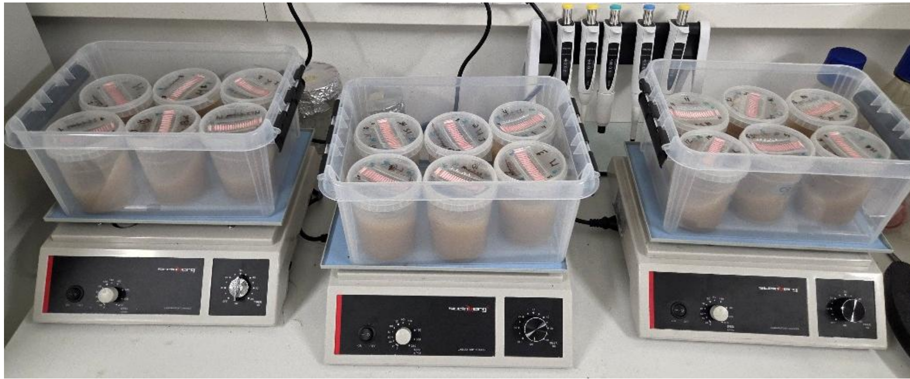


Figure 15. Experiment setup. Cultivation cups on a laboratory shaker at room temperature.

2.3 Optimization of liquid culture conditions and medium

The different parameters that were to be tested and optimized are medium composition, temperature, inoculum density, pH, duration and lighting. Different combinations of the different parameters were tested to find which factors influence growth the most and to find the optimal growth conditions.

Experiments were started by utilizing Kääpä Biotech’s liquid culture medium (Table 1) as a starting point to help identify the key growth conditions. Different treatments levels had big changes between them so that a difference could be detected and explained by the change in culture conditions.

Because fungal growth has a lot of variation, a control treatment was utilized in all the experiments which consisted of the Kääpä Biotech’s liquid culture medium, 4 % inoculum density, working volume of 250 ml, shaker speed of 140 rpm and duration of 4 days.

---

[3] Small amount was cut out from the pipette tips before autoclaving them so that the pipette tips don’t get clogged while pipetting the seed culture and to allow more even transfer of mycelium to all cups.

2.3.1 Culture conditions

Different inoculum densities (2 %, 4 % and 6 %) were tested based on inoculum densities mentioned by L.-Y. Wang et al. (2015) but were adjusted to suit the working volume of the cultivation to reach even pipetting volumes.

Cultures did not have real time pH monitoring, but the pH of the control medium was measured (Milwaukee MW101 PRO pH meter) to be 5.6 before autoclaving. The effects of pH were planned to be tested by testing pH values of 4, 6 and 8 based on Ko et al. (2017) and L.-Y. Wang et al. (2015) but due to limited time and number of treatments, only the extremes (pH 4 and 8) were eventually tested in the pH experiment. The pH of the medium was adjusted with 100 % acetic acid (17.4 M) (EMSURE®, Supelco, Merck) or 4 M NaOH to reach the desired pH value and then medium was poured into culture cups. pH values of the medium were 4.1 and 7.8 after pouring into the cups. After autoclaving and before inoculating the culture cups the pH of the medium was measured to be 4.25 and 6.1.

Temperature was to be tested in the range of 12 to 22 °C based on Ko et al. (2017) and L.-Y. Wang et al. (2015) articles. Growth box was intended to be used for the experiments to control and test different temperatures, but the liquid cooling system could not cool the box temperature. This meant that the experiments had to be performed at room temperature and a vital parameter for growth could not be tested.

In *Cordyceps militaris* liquid cultures the effects of light have been studied by Ha et al. (2020) to see if it affects the mycelium and cordycepin formation. In this study we wanted to see if light also affects *O. sinensis.* In the lighting experiment two different conditions were used: control treatment with 0 hours (0 h) of light cultivated in room temperature and a treatment with 12 hours (12 h) of light cultivated inside the growth box (contains LED lights that work on a timer) (Figure 16). The problem in this setup was that the temperature inside the growth box could not be controlled, meaning that the treatments were grown in different temperatures[4].

[4] Temperature inside the box kept increasing during the cultivations when the lights were on without working temperature control.

Figure 16. Setup for the lighting experiment. Light treatment cultured inside a growth box with 12 h of light.

2.3.2 Medium

The medium composition was optimized by changing the concentration of one medium component at a time to observe how the change in concentration affects the growth. Based on each component's original concentration (Table 2) it was decided how big changes are made. The tested changes were ½ -, control, 1.5-, 2- and 3- times the original concentration of the component listed in Table 2. Intention was to see if the added amount of the component boosts the growth or the lower amount hinders it.

After individual component concentration changes were tested, a combined effect testing with yeast and glucose was done. For this experiment the 3x yeast treatment medium (9 g/L of yeast flakes) was used as a control and for the test treatments yeast concentration was kept constant and glucose concentration was changed to ½x, 1x (control, 28.6 g/L of glucose) and 2x amount of glucose. This was done to see if the change in the ratio between glucose (carbon source) and yeast (nitrogen source) (glucose to yeast ratio[5]) influences the mycelium formation (Table 3). Yeast amount was kept constant (9 g/L) so that the glucose to yeast ratio would be smaller. If 2x glucose had been kept constant the glucose to yeast ratio would have increased, making the effects of yeast harder to detect (Table 3). The cultivation time was 5 days instead of 4 days.

[5] Not the same as carbon to nitrogen ratio (C:N). To determine C:N ratio malt extract would also need to be considered to determine true carbon and nitrogen amounts in the medium.

Table 3. Glucose to yeast ratios.

Glucose to yeast ratio (times more glucose)	Glucose (g/L)	Yeast extract (g/L)	Treatment
10:1 (≈10)	28.6 ≈30	3 g/L	Control
5:3 (≈1.7)	15	9	1/2x Glucose + 3x Yeast
10:3 (≈3.33)	30	9	1x Glucose + 3x Yeast
20:3 (≈6.67)	60	9	2x Glucose + 3x Yeast
40:1 (≈40)	60	1.5	2x Glucose + 1/2x Yeast

2.4 Harvesting mycelium, determining mycelium dry weight and wet weight

Mycelium was harvested by filtering the liquid culture through a weighed coffee filter paper (1x4, Iisi) utilizing a coffee filter cone (Nordiska Plast) (Figure 17). Filtered mycelium was washed with RO water prior drying in dehydrator (Klarstein Pro series) at 50 °C until the change in weight between two weighting points was under 10 mg (on average for 18 h). To determine the mycelium dry weight (DW) Equation 1 was used.

Figure 17. Setup for the harvesting of mycelium.

$$DW\ (g) = (filter\ paper + dry\ mycelium)(g) - (empty\ filter\ paper)(g) \qquad (1)$$

During the project it was noticed that sometimes samples reacted differently in the dryer resulting in the mycelium getting stuck to the filter paper, so the wet weight (Ww) of the filtered mycelium started to be weighed (Equation 2) to see if there is correlation between the wet and dry weight of the mycelium so that the wet weight could be used to estimate the dry weight. After collecting wet and dry weight from samples of three different experiments the results were plotted as wet weight on x-axis and dry weight on y-axis. The plot was linearly fitted.

$$Ww\ (g) = (filter\ paper + wet\ mycelium)(g) - (empty\ filter\ paper)(g) \qquad (2)$$

2.5 Bioactive compounds analysed with LC/MS

As much dried mycelium was then collected from the filter paper as possible into 25 ml Eppendorf tubes and ground into a powder. After grinding the harvested mycelium, mycelium samples from the same experiment and treatment (replicates) were combined into a pooled sample of the treatment. Then 2 g of the mycelium powder was sent for analysis to Turku Metabolomics centre.

At Turku Metabolomics center the samples were analyzed with liquid chromatograph (SCIEX ExionLC AD). Waters Atlantis Premier BEH C18 AX (1.7µm x 2.1x100mm) column was used to separate the compounds. The LC was linked with mass spectrometer (SCIEX TripleTOF 6600) equipped with DuoSpray Ion Source. The protocol screened for specific bioactive compounds found in fungi and Turku metabolic centre provided results on which of these compounds are present in the samples and what their contents are.

2.6 DNA extraction and sequencing

DNA extraction was done by using E.Z.N.A® Plant & Fungal DNA Kit from Omega Bio-tek and following their protocol. 50 mg of dried mycelium was placed into 1.5 ml microcentrifuge tube and grinded into a fine powder. Two replicates were made. Centrifuge used: Steinberg SBS-LZ-6000HS with SBS-AR-24-2 rotor (24x1.5/2ml, radius 85mm). Isopropanol (≥99.8 %, EMSURE®, Supelco, Merck) was used to precipitate DNA. When DNA pellet was resuspended, the samples were incubated in a 65 °C water bath (Steinberg systems, SBS-TWB-400) for 20 minutes and vortexed every 5 minutes. Samples were eluted twice with 100 µl of Elution buffer resulting in end volume of 200 µl. Samples were stored in -20 °C.

The extracted DNA was sent for sequencing to DNA Sequencing and Genomics Laboratory (BIDGEN) at University of Helsinki. Internal transcribed spacer (ITS) region was amplified by PCR using ITS-1F and ITS-4R primers (Table 4) and then sequenced using sanger sequencing (Sanger ABI3500xL).

Table 4. PCR primers used for ITS sequencing.

Primer	Primer sequence
ITS-1F	5’ CTT GGT CAT TTA GAG GAA GTA A 3’
ITS-4R	5’ TCC TCC GCT TAT TGA TAT GC 3’

DNA Sequencing and Genomics Laboratory provided the sequencing results as FASTA files. The resulting FASTA files were analysed by aligning the forward and reverse sequences with BioEdit and the resulting sequence was then analyzed with NCBI nucleotide BLAST to identify the fungal strain (Altschul et al. 1990).

2.7 Statistical and data analysis

All experiments had at least three replicates and results are presented as median ± standard deviation (SD). The number of replicates were increased during the project when it was noticed that there is a lot of variation in the growth. The data procured from the experiments were analyzed with one-way ANOVA and statistical significance was determined with Welch's t-test and Student's t-test.

3 Results and discussion

3.1 Effect of culture conditions on mycelium

3.1.1 Duration

The aim of the first experiment was to determine how *O. sinensis* behaves in a liquid culture and how culture time affects mycelium formation. The cultures were grown for 3, 4, 5 and 6 days. Instead of loose dispersed mycelium, typical for filamentous fungi, the liquid cultures formed round smooth mycelium pellets (Figure 18).

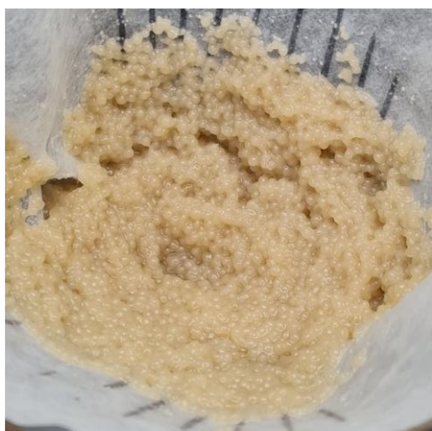


Figure 18. Mycelium pellets.

Culture time influenced mycelium formation. Statistically significant difference was found between 3- and 5-days cultures ($p \leq 0.01$) and between 4- and 5-days cultures ($p \leq 0.05$) (Figure 19). Culture time of 5 days resulted in the highest mycelium formation (8 g/L) (dry weight). Despite 5 days being the most optimal for growth, culture time of 4 days (6.3 g/L) was chosen to be used as a control treatment in the following cultures due to scheduling reasons.

The generation of biomass shows similarities to Ko et al. (2017) study (temperature 16 °C, pH 6.2, Fungal minimal medium) where culture time of 3, 4 and 5 days generated around 5 g/L, 7 g/L and 9 g/L, and in this study 5.6 g/L, 6.3 g/L and 8 g/L respectively. Ko et al. (2017) observed highest biomass generation on culture time of 8 days (13 g/L) compared to in this study the best results were from culture time of 5 days. As can be seen from Figure 19, biomass generation in 6-day cultures seems to be less than in 5-day cultures but the results have a lot of deviation due to uneven growth which can distort the results and not show the true maximum. To see if 6-day cultures generate truly less biomass or if it is better than 5-day culture it is needed to increase the duration of the cultivation in the future, for example

observe duration of 5 days to 8 days. However, even from these results it can be seen that culture time has an significant effect on biomass formation.

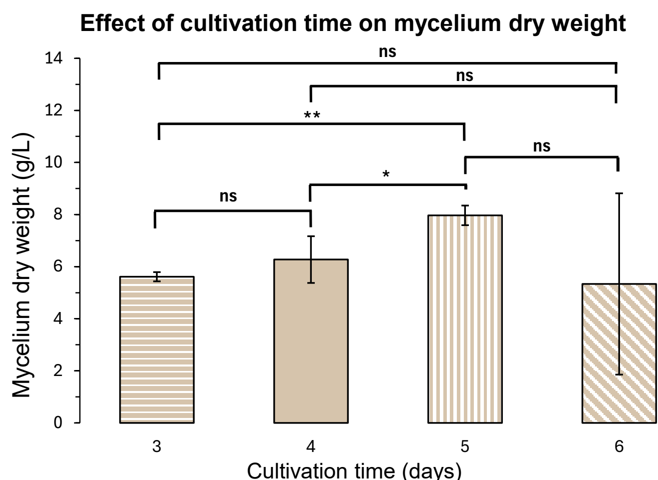


Figure 19. Effect of culture time on mycelium formation (Median ± SD, n=3). Statistical comparison with one-way ANOVA, Welch's t-test and Student's t-test. Ns=not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

3.1.2 Inoculum density

Different inoculum densities (2 %, 4 % (control) and 6 %) were also tested but data showed no significant differences between the treatments (ANOVA) when cultivated for 4 days. 4 % was thus kept as the inoculum density in all the next experiments and treatments. In this experiment all the treatments had lot of variation between the replicates (Figure 20). The growth was poor, especially in the control treatment which has been seen to generate more biomass during other experiments. The poor growth might be due to uneven transfer of inoculum from the seed culture or contamination.

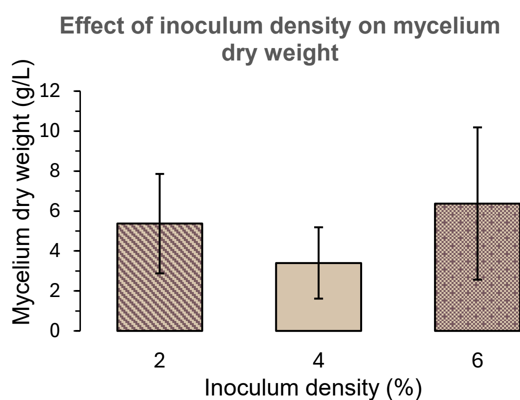


Figure 20. Effect of inoculum density on mycelium formation (Median ± SD, n=3).

Due to the growth being poor this time and the control treatment performed worse (4 g/L) than in the duration experiment (6.3 g/L) and there being no statistically significant differences present it is hard to make any conclusions on the effect of inoculum density on biomass formation. In the study by L.-Y. Wang et al. (2015) they saw that inoculum density of 3 % performed best and the lower and higher inoculum amounts generated less biomass which is completely opposite to the results in this study. To obtain more conclusive results the experiment should be repeated by using more replicates.

3.1.3 Light

Visually in the light experiment, the culture cups of both treatments looked similar (dense, pellet structure visible) (Figure 21A & 21D). After filtering differences were noticeable, mycelium from the control treatment was light, round pellets and mycelium from the 12 h light treatment contained smaller, darker pellets and less in volume (Figure 21B &21E). The dried mycelium of the control treatment (Figure 21C) was lighter in color and harder than mycelium from the 12h of light treatment (Figure 21F), which was darker and more brittle, but that could be due to the smaller amount of mycelium formed in that treatment (Figure 22).

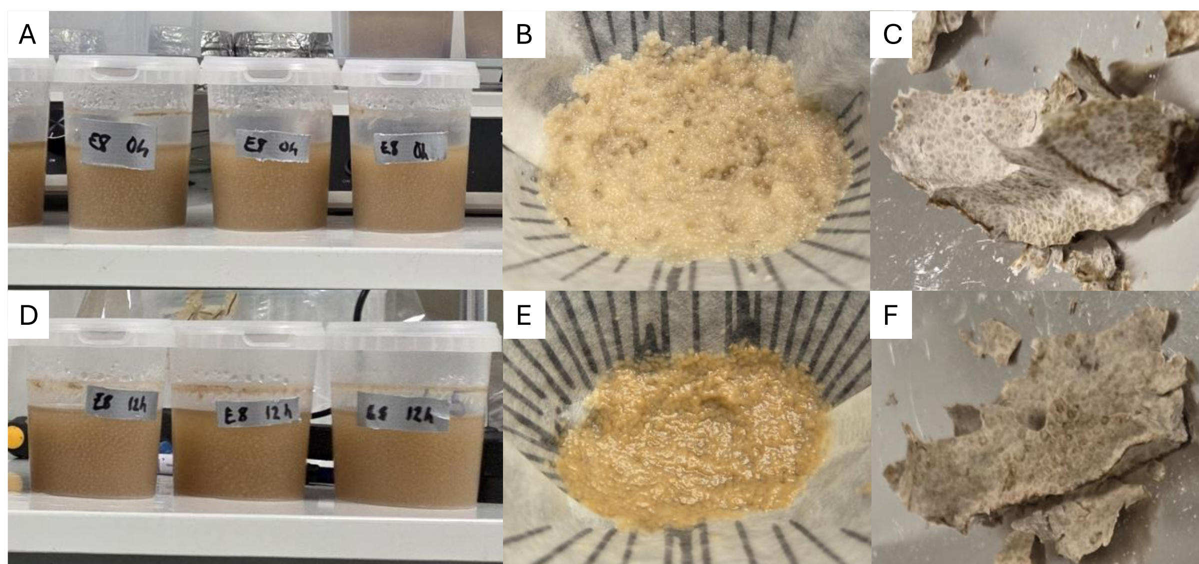


Figure 21. Visual look of the mycelium in two different light treatments. Upper row: 0 h of light (Control) – (A) liquid culture after 4 days, (B) filtered mycelium, (C) dried mycelium. Bottom row: 12 h of light – (D) liquid culture after 4 days, (E) filtered mycelium, (F) dried mycelium.

The experiment revealed that the mycelium formation in the 12 h light treatment (2.6 g/L) was significantly less ($p<0.01$) than in the 0h light (control) treatment (6.3 g/L) (Figure 22). The reason for significantly less growth in the 12 h of light treatment could be due to temperature instead of light that was studied. The 12 h of light treatment was cultivated inside the growth box in which temperature kept rising to 26 °C, compared to the control cultivated

at room temperature. It is said that the growth of the species stops at temperatures over 25 °C (X. Li et al. 2019; Lo et al. 2013).

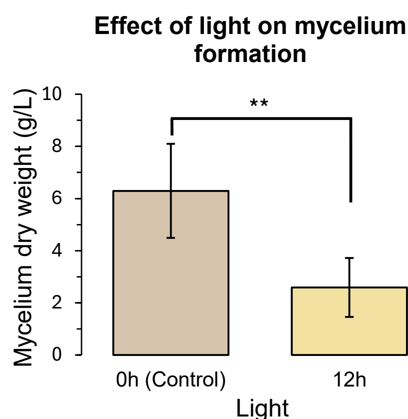


Figure 22. Effect of light treatment on mycelium formation. (Median ± SD, n=6). Statistical comparison with one-way ANOVA and Welch's t-test. ** $p \leq 0.01$

The effects of lighting have been previously studied on *Cordyceps militaris* but not that much on *O. sinensis*. In the study by Ha et al. (2020) about light conditions for *Cordyceps militaris* growth they saw increased biomass generation with the increased lighting conditions which did not happen here with *O. sinensis*. One article was found where the effects of light were studied on *O. sinensis* by Dong and Yao (2011). In that study the 0h light and 12 h light experiments reached mycelium dry weight of 18.4 g/L and 13.2 g/L respectively after 40 days culture (Dong and Yao 2011). Even though the general culture conditions were different to this study the trend was similar, when *O.* sinensis is cultured in 12 h light the mycelium formation is less than in 0 h light cultures. However, to draw better conclusions on the effect of light on *O. sinensis,* it is necessary to test the effects of light when temperature can be controlled and kept in a suitable range for *O. sinensis* growth and all the treatments can be grown in the same temperature.

3.1.4 pH

During the pH experiment, the first thing noticed was the color difference in the medium after adjusting it to pH 4 and pH 8 (Figure 23A). Medium having a pH value of 4 (Figure 23A, bottle on the left) was lighter and more yellowish compared to the pH 8 medium (Figure 23A, bottle on the right) which was darker in color. The color difference was visible throughout the culturing (Figure 23B). More distinctive differences between the treatments were seen in the mycelium structure. The pH 4 treatment resulted in smaller/finer and more filamentous/loose

mycelium (Figure 23C&D) than the pH 8 treatment (Figure 23E) and all other treatments tested during this study, which have resulted in round mycelium pellets.

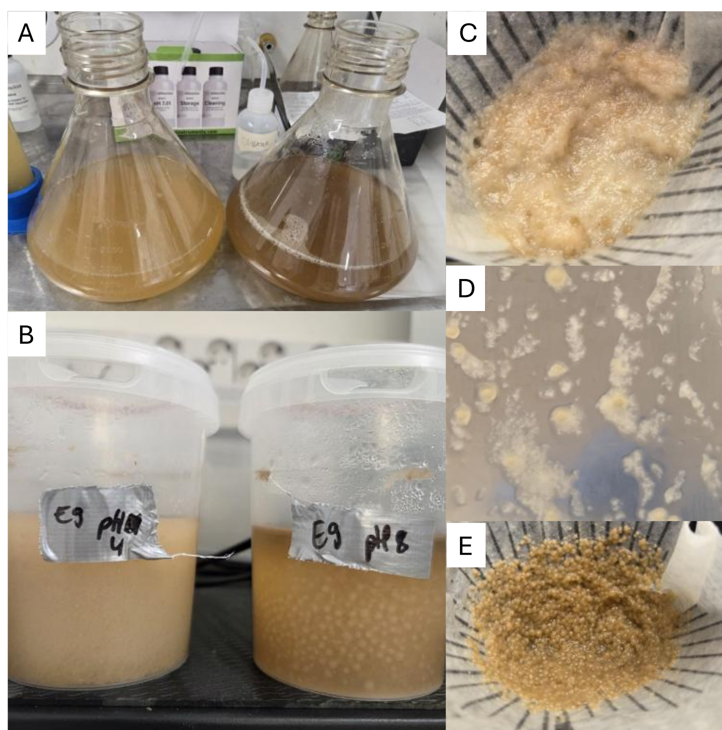


Figure 23. pH experiment. (A) pH changed the colour of the culture medium (pH 4 on the left and pH 8 on the right), (B) liquid cultures after 4 days culturing (pH 4 on the left and pH 8 on the right), (C) filtered mycelium (pH 4), (D) closeup of the mycelium on pH4 treatment, (E) filtered mycelium (pH 8).

The effect of pH on the mycelium formation was not statistically significant between the two pH treatments (Figure 24A), but the pH 4 and pH 8 resulted in dry weight of 6.3 g/L and 4.1 g/L respectively. Because real time pH monitoring was not possible in these cup cultures, pH was measured before and after autoclaving the medium and after mycelium was harvested. pH value remained more stable in the pH 4 treatment compared to the pH 8 treatment (Figure 24B). pH was almost the same after harvesting in both treatments (Figure 24B). The pH in the pH 8 treatment medium dropped from 8 to 6 during the autoclaving process, meaning that this is not true presentation on how the fungus would grow in a pH 8 medium. In the future it would be necessary to adjust the pH of the medium after the autoclaving to see how pH 8 affects the growth. But with this setup (using the culture cups) adjusting the pH after autoclaving would have introduced additional contamination risk to the process. Real time monitoring and pH adjustment systems (acid/base pumps) in bioreactors would solve this problem by allowing the adjustment of pH in real time.

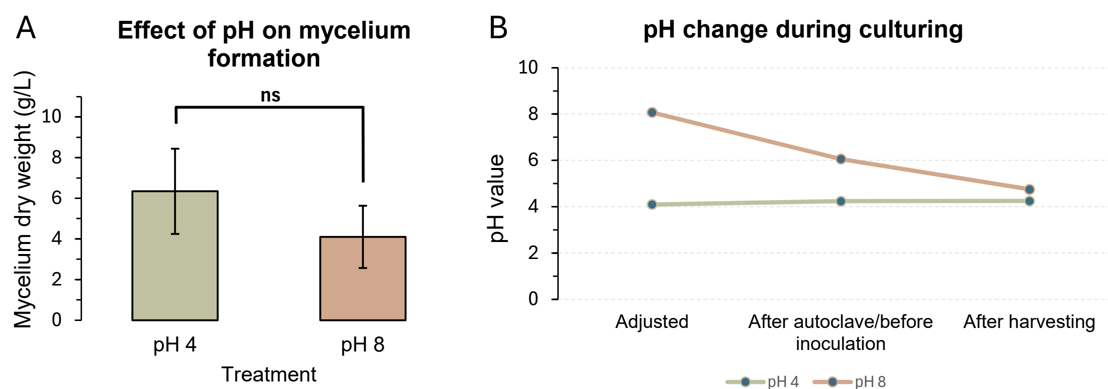


Figure 24. Comparing two different medium pH values and its effects on growth. (A) The effect of medium pH on mycelium formation (Median ± SD, n=6). Statistical analysis with single factor ANOVA (ns=not significant, p > 0.05). (B) pH of the medium during different parts of cultivation process.

Compared to literature these findings are different. For example, Ko et al. (2017) observed that biomass formation in pH 4, 6 and 8 was <2 g/L, 9 g/L and 7 g/L respectively. Whereas now culture pH of 4 performed better than pH 8 and quite similarly to how the general control treatment of this study (pH 5.6) has performed. Wang et al. (2015) also reported best growth at pH 6 but their data did not show big differences in mycelium formation in different pH conditions. The biggest difference now was seen in the fungal morphology on which pH can have an effect (Fazenda et al. 2008; L.-Y. Wang et al. 2015).

3.2 Effect of medium composition on mycelium

The effects of medium composition on the mycelium formation were tested by changing the concentration of one medium component at a time (Table 2). In the glucose experiment there were visual differences in the culture cups (Figure 25). Visually the control treatment cups looked most full and the lightest in color, which can be due to less glucose being left in the medium. The 1.5x glucose treatment didn’t visually look that different from the control, only slightly darker in color (Figure 25). The 2x glucose treatment on the other hand showed a medium layer on top of the mycelium pellet, which could be due to less pellets or more dense pellets being present that sink to the bottom and do not float (Figure 25).

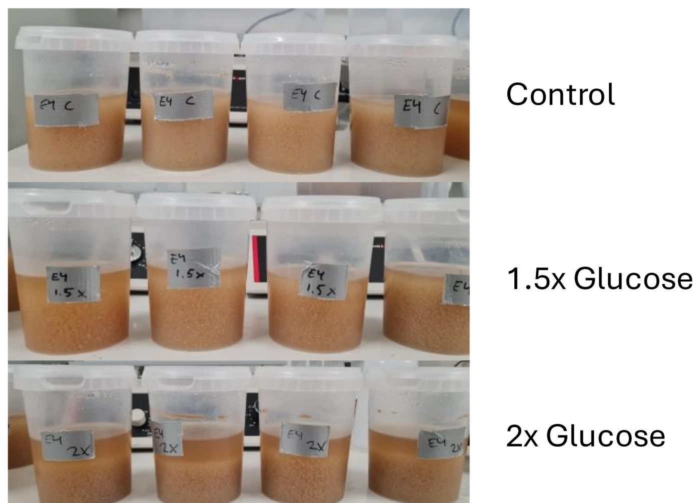


Figure 25. Treatments and their replications during the glucose experiment.

When increased malt and yeast concentration were tested all the treatments resulted in similar looking pellets (Figure 26). Based on looking at the culture cups in Figure 26, the 3x yeast treatment looked to contain the most pellets.

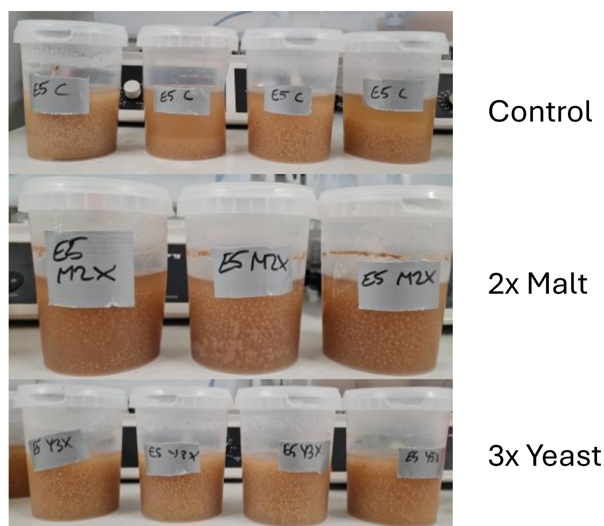


Figure 26. Treatments and their replications in the malt and yeast experiment.

Mycelium dry weight showed no significant differences between the control treatment (7.4 g/L) and 1.5x glucose treatment (7.1 g/L) but there was a statistically significant difference ($p \leq 0.001$) between the control treatment and the 2x glucose treatment (14.9 g/L) (Figure 27). One issue with the 2x glucose treatment was that it behaved differently during the drying process. The mycelium browned and formed rubbery like texture (Figure 28) instead of the light and easily breakable texture of other treatments (Figure 21C). This could be due to the mycelium having more bioactive components that started to react (for example caramelization or Maillard reaction) during the drying process. In the future the treatment

could be repeated and then tested drying the samples with few different conditions/methods to see how drying conditions affect the mycelium. Additionally, because the mycelium reacted differently and had different textures, it is not certain that the mycelium was completely dried (has low moisture level).

Mycelium dry weight showed also statistically significant differences ($p \leq 0.05$) between the control treatment (6.5 g/L) and the 3x Yeast treatment (13.3 g/L) (Figure 27). One treatment additionally focused on whether the increased amount of Malt extract would affect growth, but the data showed no statistically significant differences between control and 2x Malt treatment (Figure 27).

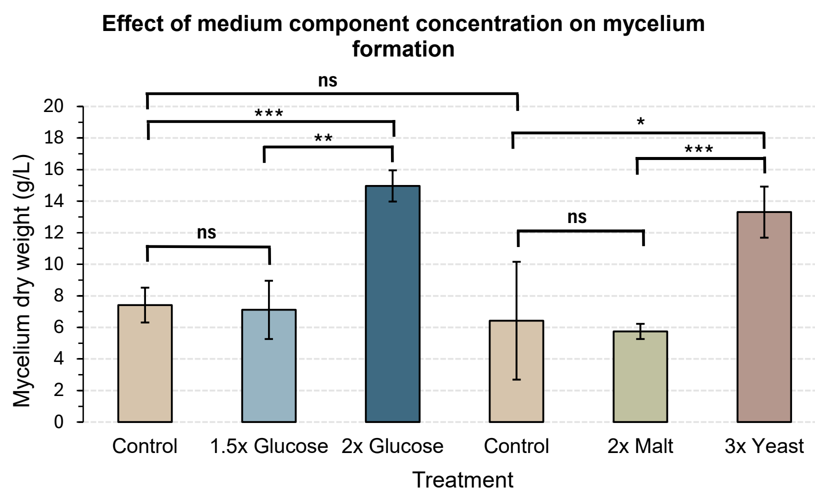


Figure 27. Effect of medium component concentration on mycelium formation after 4 days of cultivation (Median ± SD, n=3-4). Statistical comparison with single factor ANOVA, Welch's t-test and Student's t-test. Ns=not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 28. Dried mycelium from the 2x glucose treatment

Here it was seen that individually increased glucose and yeast concentrations had the biggest effect on mycelium by boosting its formation. After that it was tested how combining the yeast concentration from the 3x Yeast treatment with changing glucose concentrations would affect the mycelium. This was done by using the increased yeast concentration (3x) as a constant and changing glucose concentration in the range from 1/2x to 2x. The data (Figure 29) suggests that the mycelium formation increases when glucose concentration increases but

data showed no statistically significant differences between the tested treatments. While harvesting the 2x Glucose (+3x Yeast) treatment, few of the replicates had to be discarded because the mycelium could not be harvested by filtration. This left 3 replicates to be used for the data analysis. Data analysis showed that there is variation between the 3 replicates. The poor filtration and variation in growth in this treatment could be a result of too much sugar in the medium or contamination hindering the growth.

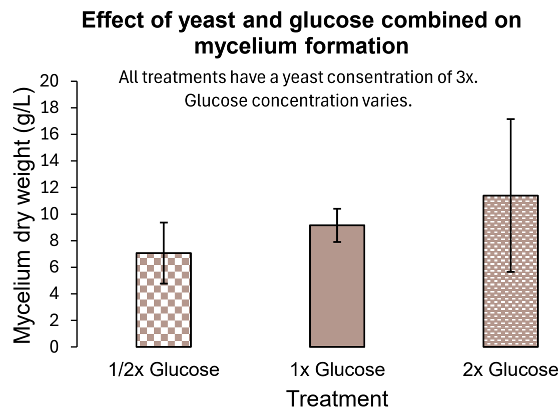


Figure 29. Combined effect testing of yeast and glucose. Yeast concentration (3x) kept as a constant while glucose concentration varies (1/2x, 1x and 2x) (Mean ± SD, n=5, 4 and 3).

To better see and understand the differences between the treatments they would need to be tested again by using multiple timepoints (for example 2-, 4-, 6- days) instead of one (cultured for 4 days) and try to mitigate the variation in the growth and possible contamination so the data would represent the treatment better and all replicates could be used for the analysis.

Due to the culture medium being different to those used in other studies it is hard to compare these findings to others. In the future, medium optimization could be continued further by modifying the current medium or testing a new medium altogether. Additionally, analyzing residual sugars in the medium would give information whether the fungus has used all the sugars provided or not, informing whether the medium has too much or too small amount of sugar available.

3.3 Variation between replicates vs between experiments

During the experiments it was noticed that there is variation between the replicates of a treatment and between experiments. Because of that every experiment round contained the same control treatment to allow confirming that the change in mycelium dry weight is caused by the changed variable of the treatment and not the general uneven fungal growth happening during the experiments. Due to the high variance in results between the replicates the amount

of replicates was increased from 3 to 6 in the later experiments. This was done to be able to get more accurate results and viable data from at least 3 replicates if some of the replicates fail to grow.

One-way ANOVA was used to determine how much variation there was between the control treatments of different experiments and between the replicates. The summary table (Table 5) revealed that there was variance between the replicates, and it ranged from 0.8 to 13.9. But the data showed no statistically significant difference between the control treatments of different experiments (Table 6).

Table 5. Summary table of how control treatment has behaved in different experiments.

Groups	Count	Sum	Average	Variance	SD
Control (duration)	3	20.1544	6.718133	0.808137	0.8989645
Control (inoculum)	3	12.2472	4.0824	3.786722	1.945950174
Control (Glucose)	4	30.7712	7.6928	1.430862	1.19618652
Control (Yeast)	3	21.9568	7.318933	13.93164	3.732511805
Control (Light)	6	36.8816	6.146933	3.245425	1.801506404

Table 6. One-way ANOVA of the control treatments.

Source of Variance	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between groups	26.01144	4	6.502859	1.581305	0.233808	3.11225
Within groups	57.57272	14	4.112337			
Total	83.58416	18				

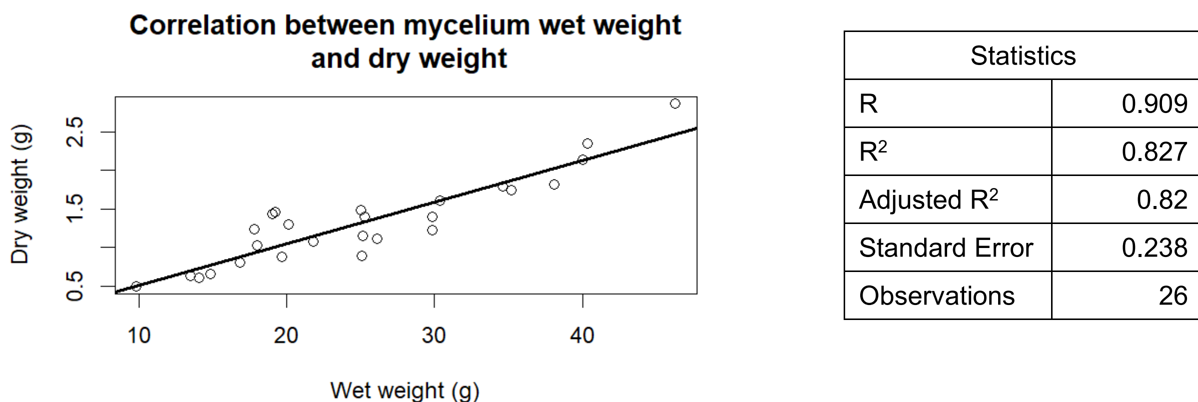
The variance in the growth between replicates could also be noticed visually when the pellet size and amount in the culture cups varied a lot. For unknown reasons not all culture cups grew equally. The uneven growth was tried to be overcome by homogenizing the seed culture and later by cutting the pipette tips wider to allow more even transfer of seed culture. These actions did not solve the problem. Contamination can be one reason for poor growth in some of the cups. If there are contaminants present the fungi fights for dominance and can in some cases lose. There are always risks of introducing contaminants into the cultures, for example even though laminar flow hoods were used, the culture cups were opened when they were inoculated. Also, the filter on the lid could malfunction, therefore preventing good oxygen

transfer or introducing contaminants into the cups during culturing. Additionally, it might not be the best suited filter for liquid cultures to allow adequate gas exchange.

3.4 Correlation between mycelium dry weight and wet weight

Due to the uneven growth and unexpected reactions during drying process the wet weight of the harvested mycelium was started to be weighted to examine if there is correlation between the dry and wet weight. This would allow estimating the dry weight of samples without the need for drying, and from samples that react differently during the drying process. However, it needs to be noted that this does not work with samples that are not filtering well. Those samples contain excess moisture from the medium that didn't flow through, increasing the weight.

Linearly fitted scatter plot of dry and wet weight is presented in Figure 30. Mycelium dry and wet weight have a correlation (R) of 0.91 and Pearson's correlation coefficient (R^2) of 0.827, these show that there is positive correlation between the wet and dry weight of the mycelium and that the wet weight could be used to estimate the dry weight by utilizing the equation of the linear fitted model (Equation 3).



Statistics	
R	0.909
R^2	0.827
Adjusted R^2	0.82
Standard Error	0.238
Observations	26

Figure 30. Correlation between wet weight and dry weight of the mycelium of 26 culture cups from three different experiments. Wet and dry weights linearly fitted.

$$DW\ (g) = 0.0539 * Ww\ (g) - 0.0282 \quad (3)$$

Wet weight could be used to estimate the mycelium dry weight of cultures without spending the time needed to dry mycelium and use this estimation with filamentous fungus that does not have direct method of estimating dry weight based on optical density like other widely used microorganisms. This could be useful in the future when process is scaled to a larger

volume because it would allow determine mycelium dry weight of the culture during sampling at different time points based on the wet weight.

3.5 Bioactive compounds in the mycelium

Prior to the experiments there was no knowledge at Kääpä Biotech about what the used strain would synthesize and to what extent. Turku Metabolic center provided results on what targeted compounds were detected from the pooled mycelium samples sent. Due to the treatments having big differences in the culture conditions and biomass formation; Control, 2x Glucose, 3x Yeast, pH 4 and 12h Light treatments were analyzed. The LC/MS analysis detected the targeted bioactive compounds typically present in *O. sinensis*; Adenosine, D-mannitol, Ergosterol, Cordycepin and Ergothioneine; and their content in mycelium varied in different treatments (Figure 31). Table 7 shows the content of adenosine, cordycepin, D-mannitol, ergosterol and ergothioneine in natural and cultured *O. sinensis* previously reported in the literature to which the detected levels from this study were compared to.

The 3x Yeast and 2x Glucose treatments generated the most biomass (13.3 g/L and 14.9 g/L, respectively) and 12h of light treatment the least (2.6 g/L) (Figure 31). But the generation of biomass is not necessarily an indicator on formation of bioactive compounds (Fazenda et al. 2008). The data was sorted based on biomass from highest to lowest.

When looking at the differences between treatments one compound at a time (Figure 31) it's seen that there is similar trend in ergosterol and ergothioneine profiles, their content increased when biomass amount decreased, finding the highest amounts in the 12h of light treatment. It has been seen that the level of ergosterol in cultured mycelium is lower than in natural *O. sinensis*, but even the highest detected level in this study (206 µg/g DW) was lower than levels previously reported in literature (380-1300 µg/g DW) (Table 7). Additionally, the content of ergothioneine in the mycelium was lower than levels previously reported in literature (9 µg/g DW versus 142 µg/g DW) (Table 7).

Adenosine content followed almost the same pattern, except for the 12 h of light treatment in which the adenosine level was similar to the control treatment even though biomass amount was lower, while mycelium from the pH4 treatment contained the most adenosine (570 µg/g DW) (Figure 31) and was the only treatment reaching adenosine levels that have been previously reported in literature (540 µg-5 000 µg) (Table 7). Compared to ergosterol, adenosine content on the other hand is reported to be higher in cultured mycelium than in

natural *O. sinensis* (Table 7). Adenosine is used in energy transfer and in DNA synthesis among other cellular functions so its content should be the highest during the growth stages (Krishna, Ulhas, et al. 2024; Chuenprasert, N, et al. 2025). The low adenosine content in the mycelium could be due to suboptimal culture conditions/medium or if the mycelium has already passed the growth stage. Testing different culture times would allow us to determine in which growth stage the mycelium is after 4 days.

With D-mannitol and cordycepin there were no distinctive pattern between its concentration and biomass amount (Figure 31). The pH 4 treatment contained the most D-mannitol (1 130 µg/g DW) followed by the 2x Glucose treatment (1 080 µg/g DW), but these levels were considerably lower than levels in cultured mycelium previously reported in literature (Table 7). Cordycepin production was the most different from the compounds in all treatments. Every other compound was present in all treatments but cordycepin was found only in three treatments from the five (3x Yeast, Control and 12 h light), although in low concentrations (Figure 31). Though the low concentrations of cordycepin can be expected because naturally *O. sinensis* does not contain high levels of cordycepin and the levels reported in cultured mycelium are also low (Table 7). The highest amount of cordycepin detected was 1.5 µg/g DW from the 3x yeast treatment which is not much and supports the values reported previously in literature (S. P. Li et al. 2006). The low cordycepin content could be due to the controversy regarding whether *O. sinensis* produces cordycepin or not, or because cordycepin biosynthesis is linked to adenosine (adenosine is converted into cordycepin) (Krishna, Ulhas, et al. 2024). Because adenosine levels were also low, it is possible that all is used in the necessary cell functions, meaning that there is not enough excess that could be converted into cordycepin (Krishna, Ulhas, et al. 2024).

Overall, the detected levels in this study were lower than previously reported in cultured mycelium even though efforts were made to increase them. This might be due to the culture conditions not being optimal for the synthesis of bioactive compounds, but also because the strain was revived from older samples that could have lacked vigor. Overall, the used strain might be poor to produce bioactive compounds. Compared to the control treatment (starting point) increased and decreased values were detected of the bioactive compounds (Figure 31). The treatments that produced the least biomass had generally higher content of bioactive compounds from these five analyzed treatments (Figure 31). The control treatment seemed to be the middle ground between biomass and bioactive compound formation. Although, in the pH4 treatment the biomass amount was slightly lower compared to control treatment;

however, it had higher content of bioactive compounds. That could have been due to the different morphology of the fungus in the control and pH 4 liquid culture treatments (pellet vs looser mycelium). More experiments should be done with the pH 4 culture to see if the change in mycelium structure is the reason for differences in chemical composition.

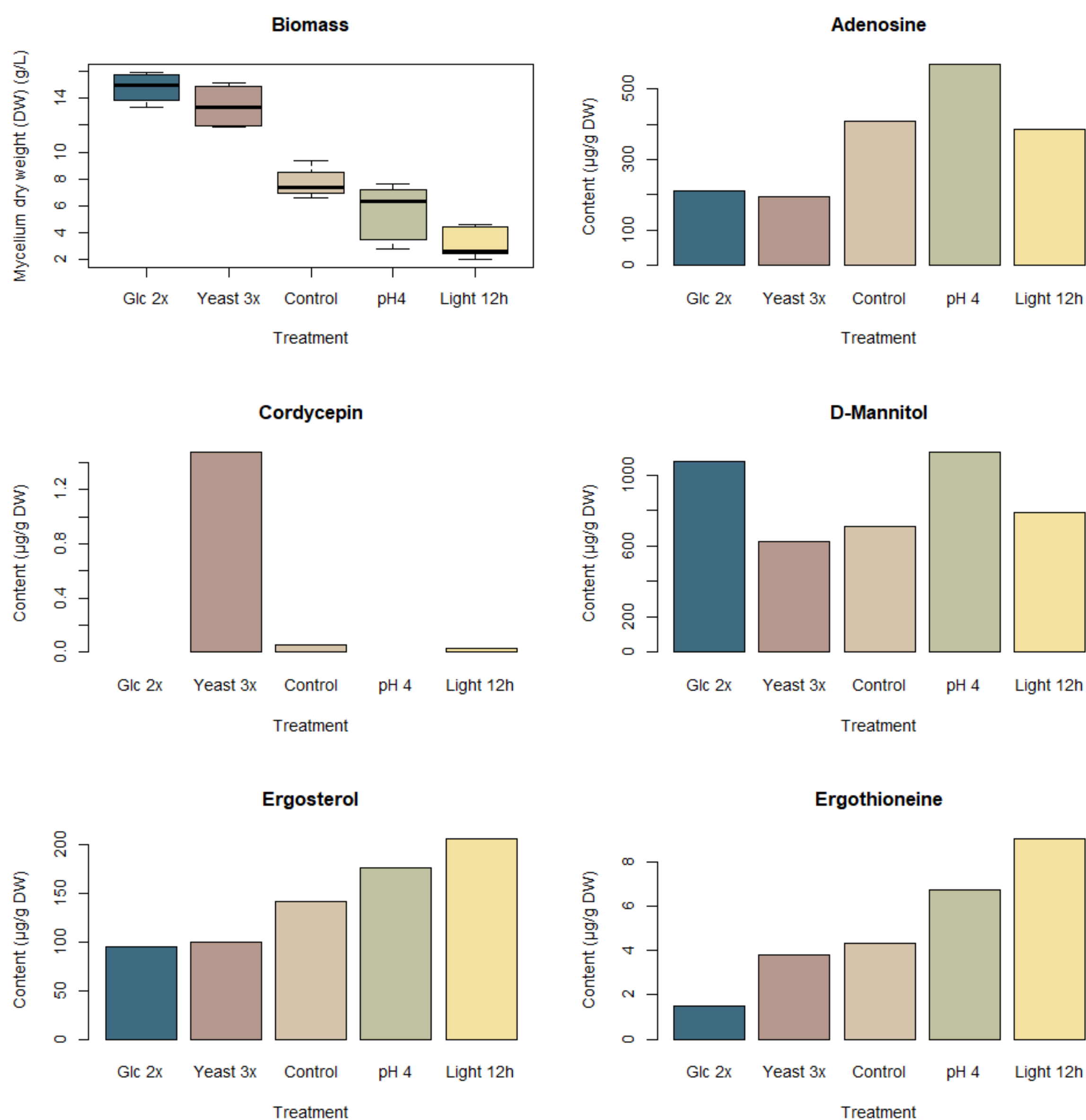


Figure 31. Mycelium dry weight (DW) and bioactive compound content of five different treatments. Data ordered based on biomass from highest to lowest. Control: pH not adjusted (around 5.6), 0h of light. Number in the yeast and glucose (Glc) treatments tells how many times more of that compound is in the medium compared to the control treatment.

Table 7. Bioactive compounds and their content in natural and cultured *O. sinensis* previously reported in literature.

Bioactive compound	Natural sinensis (µg/g DW)	Cultured sinensis (µg/g DW)	Reference
Adenosine	250-960	540-5 000	(C. Li et al. 2006; S. P. Li et al. 2006; J. Wang et al. 2015; F. Q. Yang et al. 2010)
Cordycepin	ND[a]-60	ND-35.4	(S. P. Li et al. 2006; J. Wang et al. 2015; Yan and Wu 2014; F. Q. Yang et al. 2010)
D-Mannitol	9 850-38 000	4 700-13 400	(S. P. Li et al. 2006; J. Wang et al. 2015)
Ergosterol	3 650-10 300	380-1 300	(S. P. Li et al. 2006)
Ergothioneine	-[b]	142	(Chen et al. 2012)

[a] ND=Not detected

[b] No source found

To be able to draw better conclusions about the differences in chemical composition between treatments more samples would need to be analysed. The individual replicates (not the pooled sample) would need to be analyzed to be able to determine if there are significant differences in chemical composition between the treatments.

It is also typical for macro fungi to secrete/excrete metabolites out of the cells, so in order to know the full bioactive component profile the filtered fermentation medium would need to be analysed to see if the fungus has secreted/excreted some of the relevant compounds (El-Enshasy 2007; Fazenda et al. 2008). That would give information on whether focus needs to be also put into the utilization of the filtrate in addition to the mycelium. Additionally, focusing on the metabolic pathways of the key compounds could reveal potential ways to increase the formation of bioactive compounds during liquid fermentation.

For it to be financially viable to produce mycelium on an industrial scale, the levels for the metabolites would need to be at least in the range of what has been previously reported in literature and at the same time generate enough harvestable biomass. More optimization is needed to reach that point. Increasing the cultivating time and analyzing multiple timepoints in the future would additionally give better information on the growth rate of the fungi and what is really the optimal duration for biomass and bioactive compound formation. For example, whether longer culture time is needed for the fungus to produce more of these compounds or not. In addition, testing the effect of the parameters that could not be tested in this study (for example temperature) would be needed to see how those affect the biomass and bioactive compound formation to find the most optimal culture conditions. In general, the strain utilized in liquid fermentation influences what compounds are synthesized and in what

content and which conditions are optimal for growth. It might be beneficial to compare this strain to some other strain in the future to see if the current strain is the cause for low metabolite synthesis or the culture conditions.

3.6 DNA sequencing

DNA sequencing was done to confirm that the used fungal strain is *O. sinensis.* DNA Sequencing and Genomics Laboratory (BIDGEN) at University of Helsinki provided the sanger sequencing results as forward and reverse sequence files. After aligning forward and reverse sequences with BioEdit the aligned sequence was BLASTED (www.blast.ncbi.nlm.nih.gov**)** using search set: Cordyceps sinensis (Taxid:72228)) (Altschul et al. 1990). The BLAST results showed that the identity match to *O. sinensis* strain A18 was 94.3 % (GeneBank sequence EF488439.1) (Figure 32).

```
Score            Expect  Identities      Gaps         Strand
776 bits(420)    0.0     481/510(94%)    6/510(1%)    Plus/Plus

Query  1    ACCAGCGGAGGGATCATTACAAGAAGCCGAAAGGCTACTTAAAACCATCGCGAACTTATC  60
            |||||||||||||||||||  |||||||||||||||||||||||||||||||||||  ||
Sbjct  37   ACCAGCGGAGGGATCATTATTAGAAGCCGAAAGGCTACTTAAAACCATCGCGAACTCGTC  96

Query  61   CAAGTTGCTTCGGCGGCGCGGCTCCCCTCACGGGGG-ACCGCAGCCCCCGCCTCTCAGGA  119
            ||||||||||||||||||||||  ||||||||||||  |||||| ||||||||||| |||
Sbjct  97   CAAGTTGCTTCGGCGGCGCGGCCTCCCTCACGGGGGCGCCGCAG-CCCCGCCTCTCCGGA  155

Query  120  GGTAAGGGGCAGCCGCCGGAGGTACGAAACTCTGTATTATAGTGGTATCTCTGAGTATAA  179
            |||  |||||  ||||||||||||||||||||||||||||||||| ||||||||||| ||
Sbjct  156  GGTGTGGGGCGCCCGCCGGAGGTACGAAACTCTGTATTATAGTGGCATCTCTGAGTAAAA  215

Query  180  AACAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  216  AACAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC  275

Query  240  AGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  276  AGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG  335

Query  300  CACATTGCGCCCGCTAGTACTCTAGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCA  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  336  CACATTGCGCCCGCTAGTACTCTAGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCA  395

Query  360  AGCTCTGCTTGGTGTTGGGGCTCTACGTCTGACGTAGGCCCTGAAAGGAAGTGGCGGGCT  419
            ||| ||||||||||||||||| ||||| ||| ||||||||||||||||||||||||||||
Sbjct  396  AGCCCTGCTTGGTGTTGGGGCCCTACGGCTGCCGTAGGCCCTGAAAGGAAGTGGCGGGCT  455

Query  420  CGCTACAACTCCGAGCGTAGTAATTCATTATCTCGCTAGGGAAG-TGTGGCGTTCTCCAG  478
            |||||||||||||||||||||||||||||||||||||||||| | || ||||  |||| |
Sbjct  456  CGCTACAACTCCGAGCGTAGTAATTCATTATCTCGCTAGGGACGTTGCGGCGCGCTCCTG  515

Query  479  CCGTTAAAGACCCCATCTTTAAC-CAAGGT  507
            ||||||||||||  ||||||||| ||||||
Sbjct  516  CCGTTAAAGACC--ATCTTTAACTCAAGGT  543
```

Figure 32. NCBI nucleotide BLAST alignment of our sequence to *O. sinensis* strain A18.

Because the identity match was not near 100 %, the sequence was BLASTED again without search set restrictions. Those results showed 100 % identity match to genus *Coniochaeta* (Fungi belonging to the same class as *O. sinensis* (NCBI taxonomy browser) (Schoch et al. 2020)).

Based on the sequencing results it can not be confirmed that the utilized strain was *O. sinensis.* These inconclusive results can be due to several factors. One being that the

sequencing region was 507 bp fragment of the ITS region and not the whole genome sequenced. Second, some cultures have been contaminated during this study, so the matched species could be the contaminant that has been present, hindering the growth and now distorting the sequencing results. Third, the whole ITS region was targeted so Sanger sequencing was used, which has been said to be the common sequencing method for ITS sequencing (Bellemain et al. 2010). Downside with Sanger sequencing is that it can’t separate if a sample has multiple sources of DNA (for example contamination) like high-throughput/Next-generation sequencing (NGS) methods could have (Bellemain et al. 2010).

Even though the DNA sequencing results did not confirm that the strain is *O. sinensis*, the bioactive compounds present in the mycelium match the bioactive compounds produced by *O. sinensis*. If the experiments are continued further, better identification on the strain needs to be done either by using mycelium from different experiment batches, different sequencing region and/or utilizing NGS that could differentiate multiple DNA sequences from a sample. Also, the fungus should be re-plated multiple times in hopes to get pure strain if the contamination has been present already from that stage. Furthermore, if the identification results still show that the species is not *O. sinensis* a new correct strain should be procured before any further experiments are started. One concurring problem with *O. sinensis* identification is that even in scientific literature there is no clear understanding of what *O. sinensis* is and the source of the fungal material in studies can be unclear. This is because many strains have been isolated from the wild *O. sinensis* but have later been identified through DNA sequencing as different species even though morphology and chemical composition is similar to the fruiting bodies of *O. sinensis* (Ko et al. 2017; Yao et al. 2024). This could also be the case with the strain used in this study.

3.7 General discussion and future perspectives

The results of this study were and were not what was expected. It was successful to grow the fungus in a liquid medium, but the quality and reliability of the results was not as good as hoped for due to variation in the growth and lot of inconclusive results. Below it will be discussed more about what reasons might have caused the results of this study and future perspectives of the liquid fermentation of *O. sinensis.*

3.7.1 Limitations of the study

Due to the limited time and resources available, all the necessary parameters required for bioprocess optimization could not be tested. Plans were adjusted to get the best possible information out of the experiments with the time and resources available. For example, temperature could not be controlled, resulting in the growing of *O. sinensis* in room temperature, which typically is not the optimal for the growth of *O. sinensis*. A growth box was intended to be used to grow the fungus, but the liquid cooling unit of the box (Peltier plates) could not cool down the box below ambient room temperature and when the cooling unit was running it warmed the air outside of the box which was then transferred back inside the system (temperature kept rising). In future experiments temperature control is needed because it is one of the key factors for growth (Wei et al. 2021). Testing of different shaker speeds (mixing) was also left out due to the time available and because the next step would be bioreactor experiments to which it will be better suited. In a bioreactor the mixing comes from within the culture compared to orbital shakers used in bottle cultures, so the optimal mixing conditions of one method would not necessarily be the same in the other.

The process, from seed culture to harvested and dried mycelium, was time consuming and quite soon it was noticed that there is lot of variation in the growth, and not all the cups would grow or be filterable. This resulted in discarding some of the replicates and in some cases even whole treatments if there were not enough replicates for statistical analysis. To be able to get usable data the replicate number was increased and control treatments were used for every experiment round, decreasing the number of treatments able to be tested during this study. Combining these with the fact that there were no prior liquid fermentation experiments done with this particular strain by Kääpä Biotech, the optimization process didn’t get as far as hoped for.

Design of experiments (DoE) would have been a powerful tool to utilize in the optimization process. It was planned to do the optimization by utilizing orthogonal design, but because the number of treatments able to be tested decreased and between experiments the environment, such as temperature, could not be controlled, the results of DoE would have been unusable (could not distinguish if change in growth is due to the changed parameter or an outside factor that could not be controlled (for example temperature and contamination). That’s why the experiments were continued by testing how changes in one condition at a time affect the growth compared to the control treatment that was used in every experiment round.

Another limitation that could have caused the variation in the growth was the use of fungus grown on plates as an inoculum for seed cultures. The seed cultures were made prior to the experiments from different plates or the growth on the plate could have been in completely different stage if the plate had had more time to grow in between the use. This has resulted in unique seed cultures (that could also be source of contamination) that can affect the biomass formation between experiments, but the use of the control treatment allows the comparison of the possible changes different treatments cause in the same experiment round (same seed culture in all treatments and their replicates). In the future, to remove the uncertainty of seed culture a master cell bank would need to be developed to use the same source material for all liquid cultures. To develop this, a good producer strain free of contaminants and confirmed to be *O. sinensis* would be needed.

Because literature about *O. sinensis* has unclear strains (many strains isolated from *O. sinensis* fruiting bodies) and various culture conditions used, it is hard to compare findings of this study to other studies. For example, the culture durations and temperatures used vary a lot, influencing what is a general or maximum amount of biomass generated from *O. sinensis* liquid cultures. Additionally, not all studies use seed culture as inoculum thus needing longer culture time for the fungus to adapt to a liquid culture or in general longer cultivation times are utilized. For example, in a study performed by R. Li et al. (2010) maximum mycelium DW of 10 g/L was reached in a 4 day culture (different medium and temperature than this study) and Ko et al. (2017) reached 13 g/L in a 8 day culture and 9 g/L in a 5 day culture (pH 6.2). On the other hand, Dong and Yao (2005) had a culture time of 40 days, which is a lot longer than in this study and the studies by R. Li et al. (2010) and Ko et al. (2017), and reached a maximum mycelium DW of 22 g/L. In general, there is lot of variation between used strains and for these reasons, in this study the culture strategy was optimized for this particular strain.

However, ITS sequencing results failed to confirm that the strain is *O. sinensis*. These results suggest that there is a high possibility that a fungal contaminant has been present in the cultures, which might be one of the reasons for uneven growth. The setup of the experiment and sample handling could have introduced contaminants into the cultures, or there is a possibility that the strain has never been a pure strain of *O. sinensis*. These highlight the importance of good laboratory practices and that before moving forward with the optimization the current challenges of contaminants, poor growth and unconfirmed strain need to be focused on.

3.7.2 Future

If the current challenges of the inconclusive strain and uneven growth can be overcome, the next step would be testing the parameters that could not be tested during this study and to move on to small-scale bioreactor experiments. In the future it would also be beneficial to understand how culture conditions affect the growth pattern/morphology of the fungus (pellet or loose dispersed mycelium) and how the structural difference affects the biomass and bioactive compound formation. It is known that with pellet structure the substrate and air transfer differs in the outer layer of the pellet to the core of the pellet, so it would need to be studied how it will affect the desired yields (El-Enshasy 2007; Fazenda et al. 2008). In this study, the mycelium formed mainly a pellet structure. The pH4 treatment was the only one that produced a looser mycelium and not pellets. Downsides of the pellet structure might be the limitations in substrate transfer. However, it can be beneficial for the downstream applications by making the separation of the mycelium from the culture medium easier (El-Enshasy 2007; Fazenda et al. 2008). This was also noticed in this study, but additionally it might reduce the viscosity of the culture medium which could become a problem during fermentation processes in a bioreactors with filamentous fungi (El-Enshasy 2007; Fazenda et al. 2008).

There is now some data that can be used to transfer cultivation into bioreactor experiments. The utilization of a bioreactor would allow the controlling and testing of the parameters that are yet to be tested. When bioreactor experiments are reached some DoE strategy should be used to help in the optimization process by lowering the number of combinations that need to be tested. Downstream processing needs to also be considered during bioreactor experiments to develop a process suitable for the required scale. It needs to be tested for example how different drying methods and their conditions affect the composition of the mycelium and quality of the potential end products. The aim for the future is to be able to scale the production of *O. sinensis* into industrial scale to produce bulk amounts of mycelium that could then be used to produce *O. sinensis* nutraceuticals in Finland.

4 Conclusion

The aims of the study were partially met. Based on literary review the main variables affecting the growth of *O. sinensis* were identified but not all of them could be tested due to unforeseen limitations. The fungus was successfully grown in liquid medium and the effect of some of the necessary variables on mycelium and bioactive compound formation were tested.

Even though optimization didn't get as far as hoped for, statistically significant differences between treatments were detected and biomass formation was successfully increased compared to the control treatment (starting point) and reached amount comparable to literature. It was seen from the tested treatments that medium component concentration (yeast and glucose) had the most significant effects on increasing mycelium formation than the changes in culture conditions. In this study the culture time of 4 days was able to generate 2.6-14.9 g/L of mycelium depending on the culture conditions of which the upper end is in accordance with literature. The highest mycelium dry weight (14.9 g/L) was reached when culture medium contained 57.1 g/L of glucose.

The results showed that in the samples where biomass formation increased generally the bioactive compound content decreased. Based on the five analysed treatments the pH 4 treatment might be the most desired candidate for future experiments. In that treatment the mycelium dry weight was in the same range as in control treatment, but the content of bioactive compounds was higher than on the other treatments that generated more biomass. pH of 4 influenced the fungal morphology which might be responsible for the overall better content of bioactive compounds. Even though the content of bioactive compounds was increased the levels did not reach levels reported in literature, which was one of the aims of this study. Only adenosine content reached literature levels in the pH 4 treatment.

There is potential, but also many challenges that need to be overcome and further research is needed before this liquid fermentation strategy could be scaled into industrial scale to produce bulk amounts of *O. sinensis* mycelium.

Acknowledgements

This study was conducted at Kääpä Biotech, Karjalohja.

We acknowledge DNA Sequencing and Genomics Laboratory (supported by HiLIFE and Biocenter Finland funding), Institute of Biotechnology, University of Helsinki for sequencing.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) Basic local alignment search tool. *J Mol Biol* **215**:403–410. (www.blast.ncbi.nlm.nih.gov**)** (Accessed 7.7.2025)

Ashraf, S. A., Elkhalifa, A. E. O., Siddiqui, A. J., Patel, M., Awadelkareem, A. M., Snoussi, M., Ashraf, M. S., Adnan, M. & Hadi, S. (2020) Cordycepin for Health and Wellbeing: A Potent Bioactive Metabolite of an Entomopathogenic Medicinal Fungus Cordyceps with Its Nutraceutical and Therapeutic Potential. *Molecules* **25**:2735.

Azeem, U., Hakeem, K. R. & Ali, M. (2020) *Fungi for Human Health: Current Knowledge and Future Perspectives*. Cham: Springer International Publishing.

Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P. & Kauserud, H. (2010) ITS as an environmental DNA barcode for fungi: An in silico approach reveals potential PCR biases. *BMC Microbiol* **10**:189.

The Business research company (2025a), Mushroom global market report 2025: https://www.thebusinessresearchcompany.com/report/mushroom-global-market-report (Accessed 24.9.2025)

The Business research company (2025b), Cordyceps sinensis Global market report 2025: https://www.thebusinessresearchcompany.com/report/cordyceps-sinensis-global-market-report (Accessed: 24.9.2025)

Cerletti, C., Esposito, S. & Iacoviello, L. (2021) Edible Mushrooms and Beta-Glucans: Impact on Human Health. *Nutrients* **13**:2195.

Chellapandi, P. & Saranya, S. (2024) Ophiocordyceps sinensis: A Potential Caterpillar Fungus for the Production of Bioactive Compounds. *Explor Res Hypothesis Med* **9**:236–249.

Chen, S.-Y., Ho, K.-J., Hsieh, Y.-J., Wang, L.-T. & Mau, J.-L. (2012) Contents of lovastatin, γ-aminobutyric acid and ergothioneine in mushroom fruiting bodies and mycelia. *LWT* **47**:274–278.

Chuenprasert, N., Yodsuwan, N., Wei, T. & Chamyuang, S. (2025) Optimization for High-Yield Adenosine Production from Cordyceps militaris in Submerged Fermentation. *Asian J Mycol* **8(1)**: 141–155.

Demain, A. L. (1986) Regulation of secondary metabolism in fungi. *Pure Appl Chem* **58**:219–226.

Dong, C.-H. & Yao, Y.-J. (2005) Nutritional requirements of mycelial growth of Cordyceps sinensis in submerged culture. *J Appl Microbiol* **99**:483–492.

Dong, C.-H. & Yao, Y.-J. (2011) On the reliability of fungal materials used in studies on Ophiocordyceps sinensis. *J Ind Microbiol Biotechnol* **38**:1027–1035.

Doran, P. M. (1995) *Bioprocess Engineering Principles*. Chantilly, UNITED KINGDOM: Elsevier Science & Technology.

Dryden, W. A., Larsen, L. M., Britt, D. W. & Smith, M. T. (2021) Technical and economic considerations of cell culture harvest and clarification technologies. *Biochem Eng J* **167**:107892.

El Sheikha, A. F. & Hu, D.-M. (2018) How to trace the geographic origin of mushrooms? *Trends Food Sci Technol* **78**:292–303.

El-Enshasy, H. A. (2007) Chapter 9—Filamentous Fungal Cultures – Process Characteristics, Products, and Applications. In S.-T. Yang (Ed.), *Bioprocessing for Value-Added Products from Renewable Resources* (pp. 225–261). Amsterdam: Elsevier.

European Commission, Food and Feed Information Portal Database, EU Novel food status catalogue: https://ec.europa.eu/food/food-feed-portal/screen/novel-food-catalogue/search (accessed 21.8.2025)

E.Z.N.A. Plant & Fungal DNA Kit Protocol manual, January 2024 v1.0

Fazenda, M. L., Seviour, R., McNeil, B. & Harvey, L. M. (2008) Submerged Culture Fermentation of “Higher Fungi”: The Macrofungi. In *Adv Appl Microbiol* (Vol. 63, pp. 33–103). Academic Press.

Fortune Business Insights (2025), Nutraceuticals Market Size, Share & Industry Analysis, By Product Type (Dietary Supplements, Functional Foods, and Functional Beverages), Distribution Channel (Hypermarkets/Supermarkets, Convenience Stores, Online Retail, and Others) and Regional Forecast, 2025-2032: https://www.fortunebusinessinsights.com/nutraceuticals-market-102530 (Accessed 24.9.2025)

Ha, S.-Y., Jung, J.-Y., Park, J.-H., Lee, D.-H., Choi, J.-W. & Yang, J.-K. (2020) Effect of light-emitting diodes on cordycepin production in submerged Cordyceps militaris cultures. 한국버섯학회지 *J Mushrooms* **18**:10–19.

Jungbauer, A. (2013) Continuous downstream processing of biopharmaceuticals. *Trends Biotechnol* **31**:479–492.

Kityania, S., Nath, R., Das, S., Nath, D., Patra, J. K. & Talukdar, A. D. (2022) Fungi as Nutraceutical: Present to Future. In A. C. Shukla (Ed.), *Applied Mycology: Entrepreneurship with Fungi* (pp. 187–202). Cham: Springer International Publishing.

Ko, Y.-F., Liau, J.-C., Lee, C.-S., Chiu, C.-Y., Martel, J., Lin, C.-S., Tseng, S.-F., Ojcius, D. M., Lu, C.-C., Lai, H.-C. & Young, J. D. (2017) Isolation, Culture and Characterization of Hirsutella sinensis Mycelium from Caterpillar Fungus Fruiting Body. *PLoS One* **12**:e0168734.

Krishna, K. V., Balasubramanian, B., Park, S., Bhattacharya, S., Kadanthottu Sebastian, J., Liu, W.-C., Pappuswamy, M., Meyyazhagan, A., Kamyab, H., Chelliapan, S. & Malaviya, A. (2024) Conservation of Endangered Cordyceps sinensis Through Artificial Cultivation Strategies of C. militaris, an Alternate. *Mol Biotechnol* 1–16.

Krishna, K. V., Ulhas, R. S. & Malaviya, A. (2024) Bioactive compounds from Cordyceps and their therapeutic potential. *Crit RevBiotechnol* **44**:753–773.

Lam, K. Y. C., Chan, G. K. L., Xin, G.-Z., Xu, H., Ku, C.-F., Chen, J.-P., Yao, P., Lin, H.-Q., Dong, T. T. X. & Tsim, K. W. K. (2015) Authentication of Cordyceps sinensis by DNA Analyses: Comparison of ITS Sequence Analysis and RAPD-Derived Molecular Markers. *Molecules* **20**:22454–22462.

Li, C., Li, Z., Fan, M., Cheng, W., Long, Y., Ding, T. & Ming, L. (2006) The composition of *Hirsutella sinensis*, anamorph of *Cordyceps sinensis*. *J Food Compos Anal* **19**:800–805.

Li, R., Jiang, X. & Guan, H. (2010) Optimization of mycelium biomass and exopolysaccharides production by Hirsutella sp. In submerged fermentation and evaluation of exopolysaccharides antibacterial activity. *Afr J Biotechnol* **9**.

Li, R. W. S., Yang, C., Sit, A. S. M., Kwan, Y. W., Lee, S. M. Y., Hoi, M. P. M., Chan, S. W., Hausman, M., Vanhoutte, P. M. & Leung, G. P. H. (2014) Uptake and Protective Effects of Ergothioneine in Human Endothelial Cells. *J Pharmacol Exp Ther* **350**:691–700.

Li, S. P., Yang, F. Q. & Tsim, K. W. K. (2006) Quality control of *Cordyceps sinensis*, a valued traditional Chinese medicine. *J Pharm Biomed Anal* **41**:1571–1584.

Li, X., Liu ,Qing, Li ,Wenjia, Li ,Quanping, Qian ,Zhengming, Liu ,Xingzhong & and Dong, C. (2019) A breakthrough in the artificial cultivation of Chinese cordyceps on a large-scale and its impact on science, the economy, and industry. *Crit Rev Biotechnol* **39**:181–191.

Lin, S., Liu, Z.-Q., Xue, Y.-P., Baker, P. J., Wu, H., Xu, F., Teng, Y., Brathwaite, M. E. & Zheng, Y.-G. (2016) Biosynthetic Pathway Analysis for Improving the Cordycepin and Cordycepic Acid Production in Hirsutella sinensis. *Appl Biochem Biotechnol* **179**:633–649.

Liu, Yang, Wang, X., Gao, Z., Han, J. & Xiang, L. (2017) Detection of Ophiocordyceps sinensis and Its Common Adulterates Using Species-Specific Primers. *Front Microbiol* **8**:1179.

Liu, Yi, Wang, J., Wang, W., Zhang, H., Zhang, X. & Han, C. (2015) The Chemical Constituents and Pharmacological Actions of Cordyceps sinensis. *Evid Based Complement Alternat Med* **2015**:575063.

Lo, H.-C., Hsieh, C., Lin, F.-Y. & Hsu, T.-H. (2013) A Systematic Review of the Mysterious Caterpillar Fungus *Ophiocordyceps sinensis* in DongChongXiaCao (冬蟲夏草 Dōng Chóng Xià Cǎo) and Related Bioactive Ingredients. *J Tradit Complement Med* **3**:16–32.

Luo, H. & Li, Y. (2022) Downstream Processing of Medicinal Mushroom Products. In Berovic, M., Zhong, JJ. (eds) *Biochemical Engineering and Biotechnology of Medicinal Mushrooms* Advances in Biochemical Engineering/Biotechnology, vol 184 (pp. 187–218). Springer, Cham.

Nielsen, J. C. & Nielsen, J. (2017) Development of fungal cell factories for the production of secondary metabolites: Linking genomics and metabolism. *Synth Syst Biotechnol* **2**:5–12.

Rop, O., Mlcek, J. & Jurikova, T. (2009) Beta-glucans in higher fungi and their health effects. *Nutr Rev* **67**:624–631.

Schoch, C. L., Ciufo, S., Domrachev, M., Hotton, C. L., Kannan, S., Khovanskaya, R., Leipe, D., Mcveigh, R., O'Neill, K., Robbertse, B., Sharma, S., Soussov, V., Sullivan, J. P., Sun, L., Turner, S. & Karsch-Mizrachi, I. (2020) NCBI Taxonomy: A comprehensive update on curation, resources and tools. *Database (Oxford)* **2020**:baaa062.

Sen, S., Karati, D., Priyadarshini, R., Dua, T. K., Paul, P., Sahu, R. & Nandi, G. (2023) *Cordyceps sinensis* (yarsagumba): Pharmacological properties of a mushroom. *Pharmacol Res - Mod Chin Med* **8**:100294.

Sharma, A., Ranout, A. S. & Nadda, G. (2024) Insights into cultivation strategies, bioactive components, therapeutic potential, patents, and market products of *Ophiocordyceps sinensis*: A comprehensive review. *S Afr J Bot* **171**:546–570.

Shrestha, U. B. & Bawa, K. S. (2013) Trade, harvest, and conservation of caterpillar fungus (*Ophiocordyceps sinensis*) in the Himalayas. *Biol Conserv* **159**:514–520.

Strong, P. J., Self, R., Allikian, K., Szewczyk, E., Speight, R., O'Hara, I. & Harrison, M. D. (2022) Filamentous fungi for future functional food and feed. *Curr Opin Biotechnol* **76**:102729

Wang, J., Kan, L., Nie, S., Chen, H., Cui, S. W., Phillips, A. O., Phillips, G. O., Li, Y. & Xie, M. (2015) A comparison of chemical composition, bioactive components and antioxidant activity of natural and cultured *Cordyceps sinensis*. *LWT* **63**:2–7.

Wang, L.-Y., Cheong, K.-L., Wu, D.-T., Meng, L.-Z., Zhao, J. & Li, S.-P. (2015) Fermentation optimization for the production of bioactive polysaccharides from *Cordyceps sinensis* fungus UM01. *Int J Biol Macromol* **79**:180–185.

Wei, Y., Zhang, L., Wang, J., Wang, W., Niyati, N., Guo, Y. & Wang, X. (2021) Chinese caterpillar fungus (*Ophiocordyceps sinensis*) in China: Current distribution, trading, and futures under climate change and overexploitation. *Sci Total Environ* **755**:142548.

Yan, J.-K., Wang, W.-Q. & Wu, J.-Y. (2014) Recent advances in *Cordyceps sinensis* polysaccharides: Mycelial fermentation, isolation, structure, and bioactivities: A review. *J Funct Foods* **6**:33–47.

Yan, J.-K. & Wu, J.-Y. (2014) Submerged Fermentation of Medicinal Fungus Cordyceps sinensis for Production of Biologically Active Mycelial Biomass and Exopolysaccharides. In K.-Y. Paek, H. N. Murthy, & J.-J. Zhong (Eds.), *Production of Biomass and Bioactive Compounds Using Bioreactor Technology* (pp. 93–120). Dordrecht: Springer Netherlands.

Yang, F. Q., Li, D. Q., Feng, K., Hu, D. J. & Li, S. P. (2010) Determination of nucleotides, nucleosides and their transformation products in Cordyceps by ion-pairing reversed-phase liquid chromatography–mass spectrometry. *J Chromatogr A* **1217**:5501–5510.

Yao, L., Zhu, L., Chen, C., Wang, X., Zhang, A., Gao, S., Wu, J. & Qin, L. (2024) A systematic review on polysaccharides from fermented Cordyceps sinensis: Advances in the preparation, structural characterization, bioactivities, structure-activity relationships. *Int J Biol Macromol* **282**:137275.

Zhou, X., Gong, Z., Su, Y., Lin, J. & Tang, K. (2009) Cordyceps fungi: Natural products, pharmacological functions and developmental products. *J Pharm Pharmacol* **61**:279–291.

Zhu, J.-S., Halpern, G. M. & Jones, K. (1998a) The Scientific Rediscovery of a Precious Ancient Chinese Herbal Regimen: Cordyceps sinensis Part II. *J Altern Complement Med* **4**:429–457.

Zhu, J.-S., Halpern, G. M. & Jones, K. (1998b) The Scientific Rediscovery of an Ancient Chinese Herbal Medicine: Cordyceps sinensis Part I. *J Alternat Complement Med* **4**:289–303.