Norges miljø- og biovitenskapelige universitet

Masteroppgave 2023
Fakultet for kjemi, bioteknologi og matvitenskap

Fungi to leather: The use of wood-digesting fungi to produce alternative materials.

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Acknowledgements

I want to thank Mycela for supplying the fungi used in this experiment and graciously introducing me to concepts within mycelium-based materials, and the supervisors who made this project possible. With the initial challenge caused by the limited ability to cultivate the fungi, the thesis had to go through multiple changes. Nonetheless, my supervisors helped me broaden the project, and humoured my strange ideas which would result in significant progress towards a viable product.

Abstract

There is an increasing demand for ecofriendly alternative materials. One approach for new material technology may be mycelium-based materials. Mycelium is a complex network of cells formed by fungi, which has been utilized directly used as a material or used to bind composite materials together. This thesis explores multiple approaches to mycelium materials, with a focus on leather-like pure mycelium material produced by liquid incubation of *Ganoderma sessile.* It was found that layering of the mycelium could increase the thickness and strength of the mycelium sheets.

Content

Fungi in biotechnology

Currently, our economy is largely based on unsustainable practices which have caused climate change and destruction of ecosystems around the world. Unfortunately, it has proven difficult to change these practices. We are more dependent on oil as a source of energy and as raw material for products than ever. Mining for important resources such as minerals and sand leads to destruction of local ecosystems due to toxic pollutants used in the extraction and building of infrastructure required for the mining process. Even agriculture and animal husbandry may exhaust local water supply, emit large amounts of greenhouse gases, and release large amounts of pollutants in the form of pesticides, antibiotics, fertilizer runoff and more.

If we want to achieve a future in which the impacts of climate change and ecosystem destruction are limited, a far more sustainable and circular economy must be implemented. To achieve this, a mix of social changes and implementation of new technologies is needed. In this thesis, we will examine how technologies stemming from fungal biotech may provide some solutions.

The production of food utilizing yeasts is probably one of the oldest inventions we have. It is hypothesized that fermentation of fruits was already used by early hominins a million years ago, enhancing the flavor and allowing them to better digest and store foods [1]. Early forms of beer seems to have been produced and drank in ritual feasts by the Natufians 13 700- 11 700 years ago utilizing naturally present yeasts[2]. Around the same period, 14 000 to 16 000 years ago, the commonly known brewer's yeast, *Saccharomyces cerevisiae* seems to have transported from China westwards along the

Silk Road, and would become central to the production of beer and leavened bread in Europe thousands of years[3].

Modern biotechnology has developed on the same basic concepts. One example is the production of biofuels. Here, yeasts are used to degrade cellulosic material often stemming from waste products of the agricultural industry under anoxic conditions. This leads the yeast to ferment the cellulosic material, producing ethanol which may be used as a biofuel. Recently, further developments allowed the use of wastewater streams from wood-based refineries have been used to produce a high protein yield fish feed, which could be used as an alternative to fishmeal. This is done by cultivating fungi, such as *Fusarium venenatum* and *Candida utilis*, in the waste-water stream, where the fungi digest the otherwise low-value waste, turning it into fungal biomass. The fungal biomass can then be filtered and used as fish feed [4].

A second utilization of fungi has been in the production of complex molecules. An example is the cultivation of *Aspergillus niger*, a mold known for its capabilities of secreting large amounts of a variety of molecules. The mold is easily cultivated in large bioreactors, allowing for rapid and continuous growth. The initial products were organic acids, especially citric acid, an organic acid used in a wide range of foods. Further development has allowed for production of enzymes such as amylase and xylanase, enzymes used in brewing, baking, and biofuel production [5]. The fermentation processes are not limited to the production of compounds used in food but may also be used to produce pharmaceutical compounds. *Penicillium chrysogenum* is cultivated in submerged-state bioreactors, allowing for the effective production of the life-saving penicillin antibiotics [6].

A third emerging use of fungi has been in the production of fungal-based materials. These materials are based on filamentous fungi, which form large, interconnected networks of cells. Generally, two broad approaches have been attempted. The first is pure mycelium materials. In these materials, fungi are grown in a liquid or out of a solid substrate, allowing complete separation of the mycelium from the medium or the growth substrate. The mycelium may then be flattened and treated with a wide range of chemicals, including tannins to improve the structure, plasticizers to improve the flexibility, dyes to improve the appearance, and other compounds which may provide the mycelium with desired properties. While there may be multiple possible products based on pure mycelium, pure mycelium as food and as an alternative leather seems to dominate the pure mycelium conversation. Products representing this strategy are Mycoworks Reishi™[7], a mycelium based leather, Ecovative MyForestfoods [8], and Ecovative Forager [9], representing food and mycelial foam based material [10].

Fungi may also be used to produce composite material. These materials are produced by allowing the fungi to grow through a solid substrate, often forestry or agricultural waste. The mycelium partly digests the substrate, forming a dense network connecting the waste together. The next step generally consists of a heat treatment step to kill the fungi. The product is often lightweight, heat resistant, with audio absorbing properties. Composite material has mainly been used as packaging, audio absorbing panels, and insulation. One company which attempted this is Mycela, which utilized sawdust inoculated with mycelium to produce mycelium composite panels [11].

The aim of my project was to explore approaches to utilize mycelium from four polypore fungi collected in Norway and one commercial *Ganoderma lucidum* species to make a leather-like material with potential to replace, or at least supplement, real leather.

Fungi

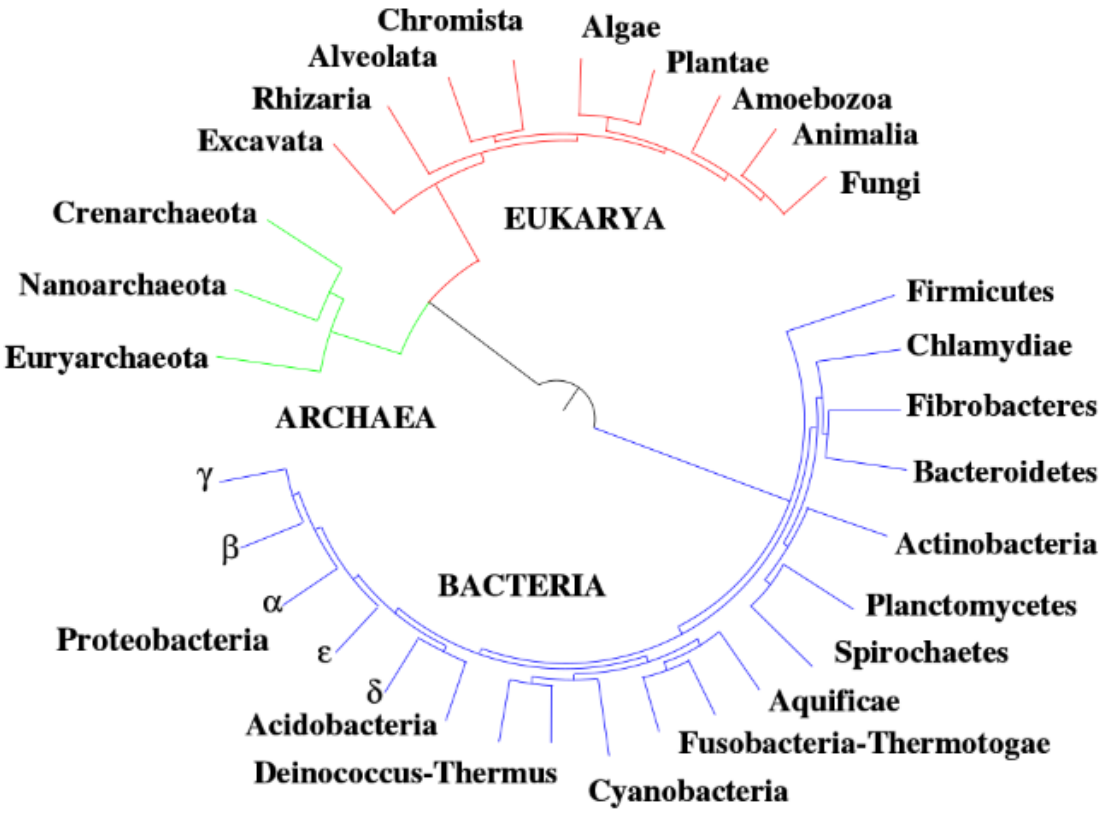

Figure 1 A phylogenetic tree of life based on fully sequenced genomes, by Franciscosp2 [12].

The kingdom of fungi is a diverse group of eukaryotic organisms. While their exact position in the tree of life is a subject of discussion, the fungi are generally placed near the kingdom of animalia, with which the fungi share many properties. All fungi are organoheterotrophic, meaning they depend on organic compounds such as polysaccharides and fats as sources of energy and precursors for anabolic processes.

The structure and life cycle of fungal organisms varies between species. Some species live as single cellular yeasts, others form complex networks of connected cells known as mycelium, and some switch between single cellular and multicellular life stages depending on the stage of their life cycle or the environment they grow in. Descriptions of fungi are therefore always met with caveats. Nonetheless, some general structures of the fungi will be introduced here, with a focus on filamentous macrofungi.

The structure of the fungal cell

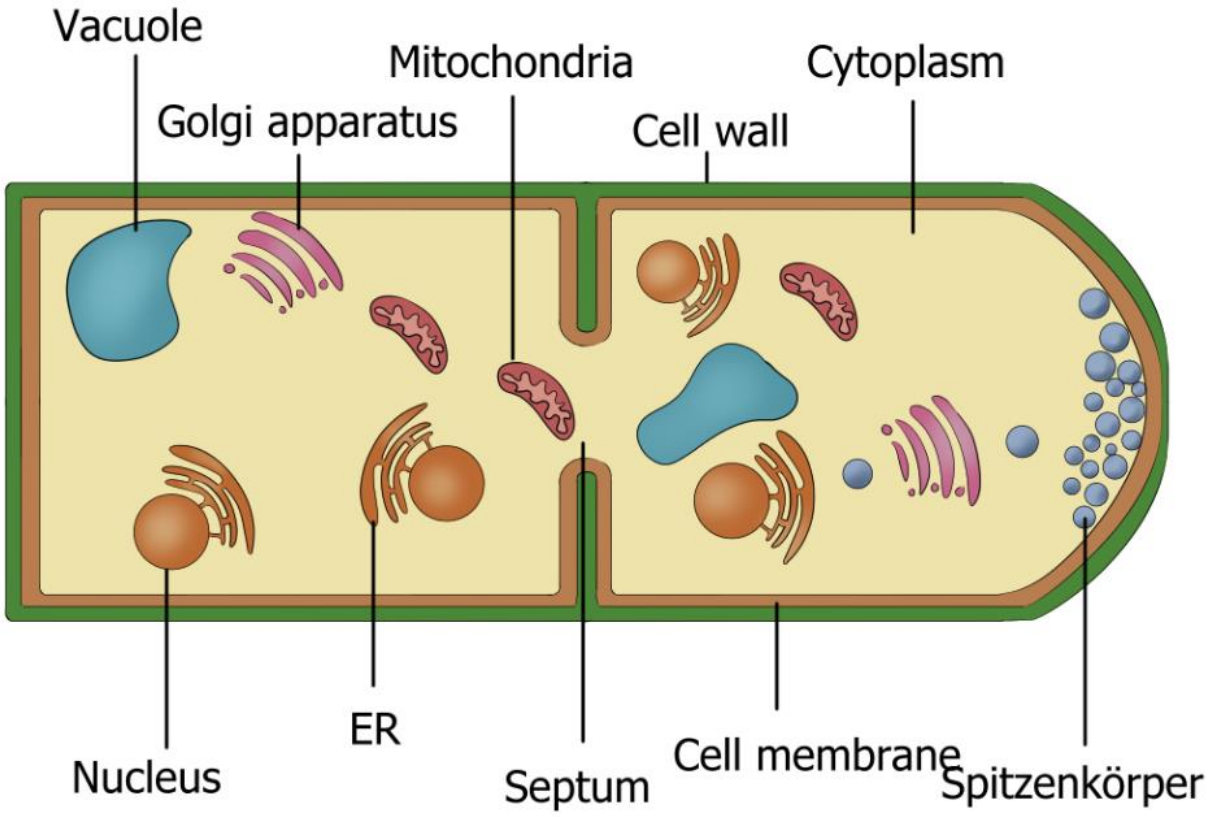

Figure 2 The general structure of fungal hyphal cells, with common intracellular components shown, including the nucleus surrounded by an endoplasmic reticulum (ER), golgi apparatus, mitochondria, vacuoles, spitzenkörper, cell walls, cytoplasm, and cell membrane.

The cytoplasm of the fungal cell is enclosed by a plasma membrane. This membrane is composed of a lipid bilayer interspersed with proteins and sterols which ensure membrane function and flexibility. An aspect that differentiates fungi from animal cells is the presence of ergosterol in the cell membrane, which has been used to detect the presence of fungi. Another aspect that differentiates fungal cells from animal cells is the presence of a cell wall outside the cell membrane. The wall is composed of long chains of carbohydrates, including geta-glucans and chitin, proteins, and more, to form a strong but flexible structure. By layering the cell wall components, the cell creates a more structural composition near the plasma membrane, and a more functional layer facing the outside, which enables the cell to better interact with the environment [13-15].

Inside the cells, much like the other members of Eukarya, fungi store their DNA in form of histone containing linear chromosomes in an organelle composed of a double layered membrane called the nucleus. The number of chromosomes and nuclei vary between species and life stages of the fungus [14, 15].

The nucleus is surrounded by a membrane network called the endoplasmic reticulum. This membrane network is largely involved in protein and lipid synthesis, especially for cell membrane and cell wall components. The products of the endoplasmic reticulum may travel to the Golgi apparatus, another

membrane complex, but this system is more involved in the modification of the lipids and proteins with polysaccharides, and the ordered transport of the newly synthesized cell components and enzymes, especially those to be secreted out of the cell. Other notable membrane structures include the vacuoles which are large vesicles involved in osmoregulation, storage of small molecules, biosynthetic precursors, degradation of molecules, and other intracellular regulation. The cell also contains smaller vesicles known as lysosomes, which are less studied but known to be involved in processes such as the degradation of organic compounds. A membrane organelle unique to fungi is the Spitzenkörper, a complex of vesicles and proteins involved in the extension and growth of hypha. The vesicles accumulate on the apex of the growing hypha, or near the cell wall in fungi spores, or at the location where the hypha branches, releasing the vesicles into the environment through exocytosis, elongating the hypha as new cell walls are built. This elongation of the hypha is what enables the fungi to produce large networks of hypha colonizing huge surfaces while also forming networks by fusing branches of the hypha to each other[13-16].

Most of them, like other eukaryotes, possess mitochondria. These are organelles which perform oxidative phosphorylation using organic compounds and oxygen as energy sources and terminal electron acceptors respectively to produce adenosine triphosphate, a highly energetic molecule the cells use to drive cellular functions. Mitochondria are effective organelles in the presence of oxygen but have evolved to live in environments where oxygen is almost entirely absent, such as in the gastrointestinal tract of herbivorous animals. As such, some fungi have become obligate anaerobes, and possess hydrogenosomes, organelles originating in mitochondria, which assist the fungi in producing ATP in anaerobic environments [14, 15, 17].

The morphology of filamentous fungi

Filamentous fungi are composed of what is known as hypha. Hyphae are long, thread-like structures composed of fungal cells, and a shared cell wall surrounding the cells. The cells inside the tubular cell wall may be separated by septa, indents of the cell wall that are perforated with pores allowing transport of cytoplasm and its components. The size of the pores varies among species, but the pores may be large enough to transport even larger cell components, including ribosomes, mitochondria, and even nuclei. This does make the concept of individual cells in the fungi a bit more fluid. This fluidity is taken to the extreme in non-septate fungi. These are fungi which produce hypha that do not have septa, which allows a flow of all the cellular components, making the hypha appear more as one massive elongated multinucleated cell instead of a chain of unique cellular units.

The network of mycelium allows the fungi to gather nutrients by releasing digestive enzymes into the environment and absorbing the nutrients set free from more complex molecules such as cellulose. Since the mycelium may stretch over out over a larger area, nutrient conditions may differ, but controlled movement of cytosol and the nutrients in it allows the fungi to shift the nutrients into resource exhausted areas. The fungi may also respond to changing conditions, such as draughts by retracting the cytosol from exhausted areas into the more nutrient rich areas, leaving a largely empty hull composed of the rigid cell wall, which may be reclaimed later in response to improved conditions [18].

One example where the interaction between varied nutrient rich areas is shown in the mutualistic relationship fungi have with plants. Plants, especially trees, are efficient producers of carbohydrates by photosynthesis, but they are often limited by their access to nutrients and moisture. Mycelium producing fungi are far better at moving through the soil and degrading matter than the trees, but as

heterotrophic organisms, are entirely dependent on obtaining carbohydrates as sources of energy and components synthesis of complex molecules. Fungi has then developed a mutualistic relationship by producing mycelium which lays in contact with the root cells of the tree, in the form of ectomycorrhiza, or even enters the root cells of the tree, in the form of arbuscular mycorrhiza. Here, the tree and fungi exchange carbohydrates produced by the tree, and nutrients and moisture the fungi obtained, benefiting them both.

Polyporales

The Polyporales is an order found in class agaricomycetes, division Basidiomycota. They are commonly known as bracket or shelf fungi, and recognizable by the often hardy basidiocarps developing on trees. Traditionally, the order was based on morphology, which is where the name, which means many pores, comes from, as many of the members shed spores from dense tubes beneath the caps. Modern phylogenetic techniques, based largely on the sequencing of select genes, including the internally transcribed spacer of the nuclear ribosome (ITS), have since provided evidence that corticoid species, fungi which develop fruit bodies resembling flattened crusts should be grouped in the same order [15]. There are currently an estimated 1800 species in order Polyporales, with significant local variation, leading to a continuous discussion of the taxonomy and a frequent discovery of new species [19].

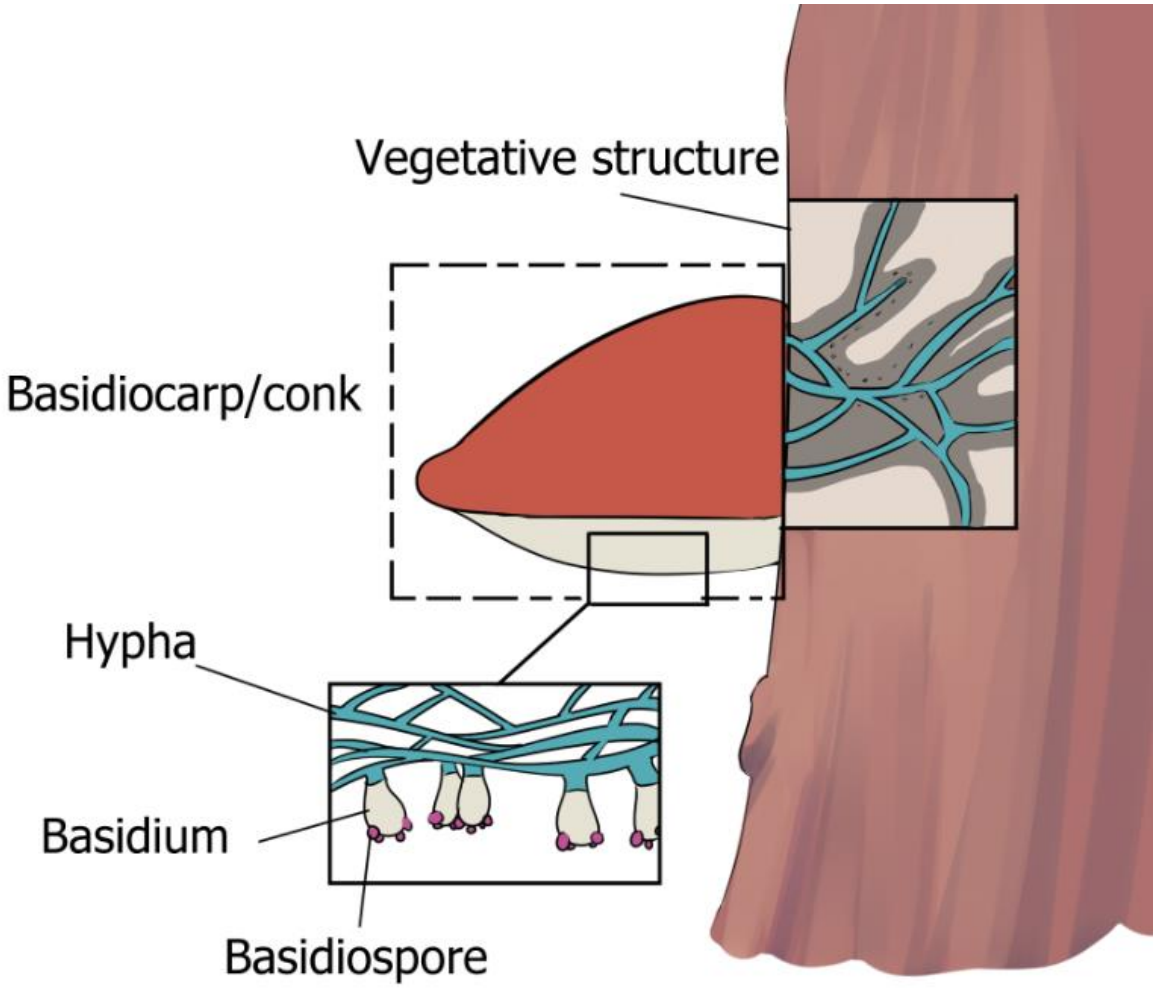

Figure 3 An illustration of a polyporale on a tree stump, with the microscopic spore bearing structures visualized. The right part of the picture illustrates the mycelium invading and decaying a wooden structure.

Their general structure, illustrated in *Figure 3* is composed of two portions, a vegetative portion and a fruit body. The vegetative portion is composed of microscopic hypha penetrating the tree. There they release enzymes involved in decomposition of the wood, releasing polysaccharides and other important compounds for the fungi to absorb. The fruitbody, often in the shape of a conk, is the method the polyporales use for spore dispersal. The fruitbody contains large numbers of pores which, each which is filled with basidium carrying hypha. Basidium are spore structures found in the basidiomycota, and produce small basidiospores to be discharged by budding, releasing them out of the pores and into the wind and weather.

The members of the Polyporales are generally saprotrophic and well known for their ability to digest wood. This is generally achieved by either utilizing brown rot or white rot degradation of the wood. Brown rot is based on the degradation of cellulose and hemicellulose in the tree, while the lignin, a complex polymer which binds cellulose and hemicellulose, is only degraded to a limited degree. This results in the distinct brownish rotten wood which breaks apart as cubes when exposed to mechanical stress. White rot is far more thorough in the digestion of the wood, attacking both the cellulose, hemicellulose, and the lignin protecting the cells simultaneously. The result is the wood taking on a white fibrous texture, which gives white rot its name [20].

Evolutionary, the brown rot fungi developed by the loss of enzymes involved in the degradation of lignin. While the selection pressure which favoured this approach is not entirely clear, some evidence suggests that this may have given the brown rot fungi some advantage as generalists. Brown rot species also seem to favour gymnosperm hosts, while white rot hosts favour angiosperms, though it should be noted, that both groups can often live in a wide range of hosts if given the opportunity [21].

In the current project, the focus lies on four Polyporales species, three of them representing white rot species, and one representative of the brown rot species.

Ganoderma lucidum

Figure 4 Picture of a Ganoderma lucidum bracket, by Klaus Høiland[22]

Ganoderma lucidum [23] (in Norwegian lakkjuke) is a white rot fungus which goes by many names, but the most common names are probably Reishi or Lingzhi. The fungus is relatively well known due to its mythical health properties, supposedly providing spiritual and physical health benefits when consumed in the form of powders, dietary supplements, or tea. None of these benefits have been proven, nor does the fungus appear to do any harm when consumed, which makes it largely unproblematic if it is not used instead of evidence driven treatment [24].

The appearance of *Ganoderma lucidum* is of that of a bracket fungus with a true stipe, concentric growth zones and a highly lacquered pileus, as seen in *Figure 4*. It is generally found on hardwood substrates in temperate regions in the northern hemisphere. It is thought to be native to Europe and Asia, though it has also been found in Utah and California in the USA, probably having been brought there by humans. It is often not possible not identify *Ganoderma* species reliably from each other utilizing only the morphology. The use of molecular techniques, generally the sequencing of the internally transcribed spacers (ITS) of the nuclear ribosomes, DNA-directed RNA polymerase II subunit RPB1 (RPB1), DNA-directed RNA polymerase II subunit RPB2 (RPB2), and Translation elongation factor 1 alpha (Tef1α) are recommended for an exact identification [25, 26].

Ganoderma applanatum

Figure 5 Picture of a Ganoderma applanatum brackest, by Klaus Høiland[27].

Ganoderma applanatum (in Norwegian flatkjuke), also known as Artist bracket, is most recognizable for its large and woody brackets. The fungi are generally saprobic white rot fungi, but may be parasitic under the right condition, mostly attacking deciduous wood. The bracket can reach up to 75 cm wide, and is concentrically furrowed with a gray, dark gray, or brown surface on top. The underside is white and known to stain brown upon touch. The fungus is found all across the world [28, 29].

The tendency to stain upon touch is where it acquired the name Artist bracket, as sketches can be made in its underside. Dried *G. applanatum* brackets are also used as flavour enhancers in Asian cousins. Otherwise, the fungi, much like *Ganoderma lucidum* is thought to provide a wide range of beneficial health effects, including anticancer, antidiabetic, and antibiotic properties, but none of these seem have been confirmed in human trials [29-31].

Trametes versicolor

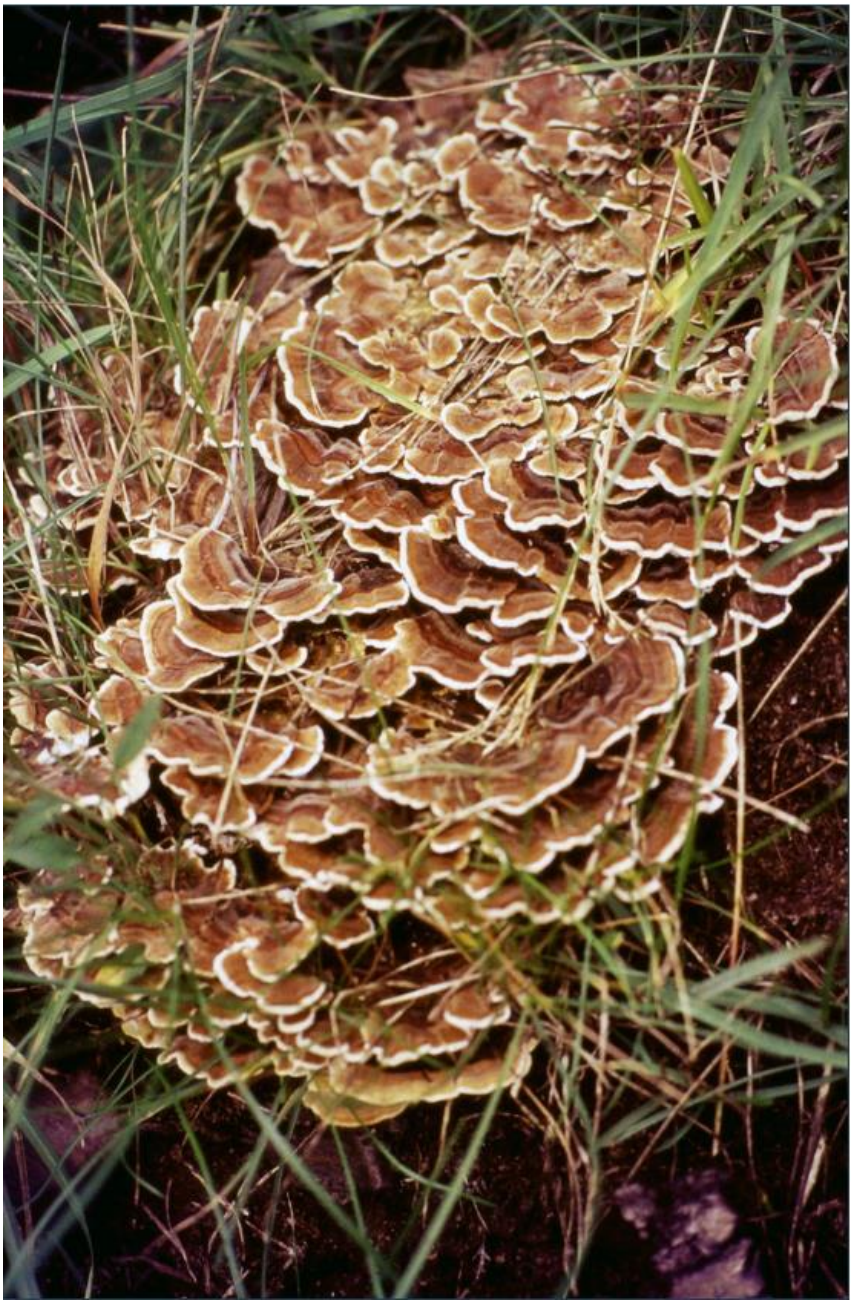

Figure 6 Picture of Trametes versicolor brackets on an old maple root, by Klaus Høiland [32].

Trametes versicolor (in Norwegian silkekjuke)*,* also known as Turkey tail is recognized by its accumulation of small brackets around 1.6 to 6.8 cm wide, and 1-3 mm thick. The brackets have multicoloured concentric zones, generally black to brownish towards the middle and transitioning to white on the edges. It is a white rot species mostly found on hardwood trees and conifers all across the world [33].

There are claims of anticancer and immunostimulant properties of certain polysaccharides produced by *T. versicolor*, but the evidence does not appear to be conclusive[34].

Fomitopsis pinicola

Figure 7 Picture of a Fomitopsis pinicola conk by Jason Hollinger (jason) at Mushroom Observer.

Fomitopsis pinicola [35] (in Norwegian rødrandkjuke) is a less known brown rot species found in the coniferous forests of the temperate regions in the northern hemisphere, mainly in Europa. Much like *G. lucidum*, it is thought to have medicinal properties, with some anti-cancer claims, but these studies have been very limited, and do not provide strong evidence for the therapeutic properties of the fungus [36].

Appearance wise, *F. pinicola* produces a large fan shaped basidiocarp, commonly known as a red belt conk. Much like *G. lucidum*, it grows by producing concentric growth zones, but may often be differentiated by the lack of a distinct stem, and a less lacquered pileus. Otherwise, confirmation of identification is recommended by sequencing of the ITS region, the RPB2, and the Tef1α [36, 37].

The unsustainable clothing business

We consume and dispose of more textiles every year. In USA alone, an estimate of 17 030 000 tons of textiles were disposed of, with 11 300 000 tons ending up in landfills [38]. There are several challenges with the synthesis and disposal of different types of textiles and leather.

Synthetic textiles

Today, an estimate of 69% of textiles are composed of synthetic fibers, which are synthesized from petroleum products. Textiles are often desired due to their wide-ranging properties, being utilized as

alternatives to leather, as lightweight sports clothing, as cleaning products and more. Unfortunately, the massive consumption of synthetic textiles has led to wide ranging environmental impacts. The synthesis of synthetic textiles relies on energy intensive processes discharging large amounts of CO^2 into the atmosphere, and produces contaminated efflux water, which is often discharged into local water systems. Washing of synthetic textiles also releases microplastics into the waterways, which is thought to have significant effects on aquatic environments, especially on invertebrates. Once they are disposed of, synthetic textiles largely end up in landfills, where they take more than 50 years to degrade, quickly filling up landfills, leaching toxic compounds and microplastics in the meantime [39-42].

Cotton

Natural fibres are not without problems either. Cotton, the most used natural fibre, requires large amounts of water to grow, and is often grown in areas which cannot supply that water to the crops without human assistance. This leads countries like China, Egypt, Pakistan, Uzbekistan, Turkmenistan, and many others to spend large amounts of water for irrigation. The most infamous consequence of this destruction of the Aral Sea. The Aral Sea was the world's fourth largest lake, before the Soviet Union decided to use the Aral Seas feeder rivers to irrigate cotton fields to provide for the Unions cotton demands. This led to a drop in volume, leading the water to become contaminated and the soil around the lake to become saline and otherwise toxic, doing immense damage to the environment and the people living there [43, 44].

The water usage of the plant during the growing season is not the only issue. Cotton plants are often plagued by pests, leading the farmers to use pesticides to protect their valuable crops. Despite cotton only covering 2.4% of the world's arable land, it accounts for 11% of the world's sale of pesticide. The pesticides and excess fertilizer are carried into local water systems [44]. This leads to disruptions in normal ecosystem functions, large algae blooms, and diminished water quality for consumption [45]. This becomes especially important as the damage caused to our water resources by cotton farming intersects with other environmental destruction and changing weather patterns due to climate change.

The processing of cotton fibers is also highly energy intensive and results in the release of pollutants. Wet processing utilizes large amounts of water and may release significant amounts of toxic effluents including heavy metals, chlorinated organic compounds, salts, surfactants and more [46].

Leather

Leather is used in the production of clothes, shoes, and accessories such as bags and belts. It is a highly desired material due to its appearance, strength, durability, and flexibility. Much like cotton though, it being natural does not mean its production is unproblematic. The preparation, tanning, dyeing, and crusting of the leather utilizes strong acids, heavy metals, chromium, synthetic tannins, oils, biocides, resins, and more to achieve the desired properties and appearance of the leather. This results in significant amounts of wastewater and non-biodegradable sludge which must be disposed of adequately [47].

One case of contamination due to leather production is the city of Kanpur, India. The fields, the water, and the air in the city of Kanpur on the Ganges have been contaminated with large amounts of Cr(IV) as a consequence of the local tanneries. This has led to an increase of cancer, respiratory tract problems, and contact dermatitis in the area, and significant ecological damage due to the toxicity of chromium.

Due to the proximity of the city on the Ganges, this damage is also not only local, but is one significant part of the pollution of the holy river [48, 49].

It should also be noted that leather is an animal product, which means the production depends on the slaughter of animals. As people have grown more aware of the mistreatment of animals in industry, the demand for cruelty free products has grown. This demand has been partly covered by polyurethane or polyvinyl chloride-based products, but since these are petrochemicals, they do often not satisfy sustainability goals. Interestingly, a new industry of so called vegan leather, utilizing plant products and fungi has developed, and grown quickly, proving the demand for cruelty free and non-petrochemical based alternatives [50, 51].

A friendlier alternative

New materials should aim to have lower impact on the environment than synthetic and traditional materials. This may be achieved through lower emissions during the production, reduced efflux release of toxic chemicals, or by the utilization of waste products, using until now unused or poorly used materials. Fungi could contribute to all these factors.

Method

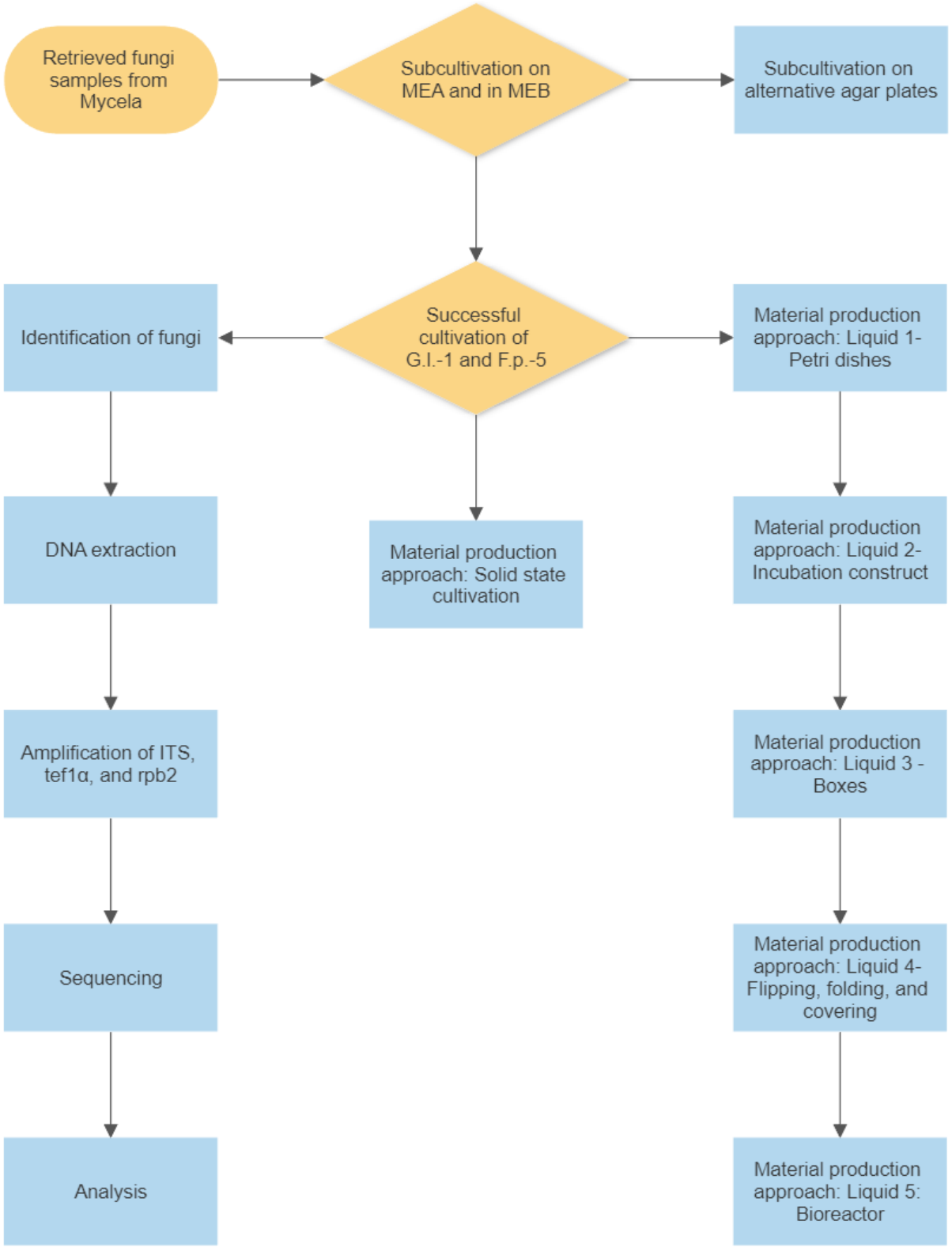

Figure 8. A flowchart of the experiments performed for this thesis, starting with the mycelium acquired from Mycela.

The experiments would explore three main areas. The identification of the cultivatable fungi using sequencing, the cultivation of the fungi in saw dust, and the cultivation of the fungi in liquid. The flowchart in *Figure 8* provides an overview of the processes.

Cultivation of mycelium

Table 1. A list of fungi on agar plates retrieved from Mycela [11].

Fungi	Abbreviation
Commercial *Ganoderma lucidum*	G.l.-1
Wild-type *Ganoderma lucidum*	G.l.-2
Wild type *Ganoderma applanatum*	G.a.-3
Wild-type *Trametes versicolor*	T.v.-4
Wild-type *Fomitopsis pinicola*	F.p.-5

Five fungal cultures were acquired from Mycela [11]. The species and their correspondent abbreviations are listed in *Table 1.*.

Figure 9. Ganoderma lucidum (G.l.-1) mycelium on malt extract agar retrieved from Mycela.

Ganoderma lucidum (G.l.-1), pictured in *Figure 9*, was retrieved as a dense white mycelium. The mycelium had a slightly dusty appearance and was strongly attached to the malt extract agar (MEA).

Figure 10. Ganoderma lucidum (G.l.-2) on malt extract agar retrieved from Mycela.

Ganoderma lucidum (G.l.-2), pictured in *Figure 10*, was retrieved as a thinner layer of mycelium that did not cover the entire plate, with some dark brown crusty sections towards the center of the mycelium. The mycelium was strongly attached to the agar.

Figure 11. Ganoderma applanatum (G.a.-3) mycelium on malt extract agar retrieved from Mycela.

Ganoderma applanatum (G.a.-3), pictured in *Figure 11*, was retrieved as a largely brown thick and dense layer of mycelium, with a thinner white layer of mycelium near the agar piece used to inoculate the plate. The mycelium could be removed from the agar as sheets.

Figure 12. Trametes versicolor (T.v.-4) mycelium on malt extract agar retrieved from Mycela.

Trametes versicolor (T.v.-4) was retrieved as a powdery white mycelium covering the entire petri dish in a fine layer. The mycelium was loosely attached to the agar.

Figure 13. Fomitopsis pinicola (F.p.-5) on malt extract agar retrieved from Mycela.

Fomitopsis pinicola (F.p.-5), pictured in *Figure 13*, was retrieved as a white, loose, and layered mycelium covering the entire petri dish.

The isolates were subcultivated on malt extract agar (MEA) (15g Malt extract [Millipore, VM999391143], 2.5g BD Bacto ™ Proteose peptone No. 3 [211693], 7.5g agar (Merck Millipore, CAS 9002-18-0), 500 ml purified H_2O, pH 5.7) and in Malt extract broth (MEB) (8.5g Malt extract [Millipore, VM999391143], 1.5g BD Bacto ™ Proteose peptone No. 3 [211693], 500 ml purified H_2O, pH 5.7). MEA plates were inoculated by transferring a 1 cm^2 piece of mycelium covered agar to the MEA plates, and the plates were incubated at 25 ± 0.25^oC (New Brunswick Innova 42R Incubator shaker series). Malt extract broth (MEB) (8.5g Malt extract [Millipore, VM999391143], 1.5 g BD Bacto ™ Proteose peptone No. 3 [211693], 500 ml purified H_2O) was transferred to sterile glass culture tubes, which were inoculated with the fungi by inoculation loops transferring tiny mycelium scratched from the agar surface, and by transferring 1 cm^2 pieces of mycelium covered agar to the tubes. The tubes were then incubated at 25 ± 0.25^oC and shaken by hand daily.

The isolates were also cultivated on potato-dextrose agar (Veterinærinstituttet, Norway), Sabourand agar (Veterinærinstituttet, Norway) and Dichloran glycerol agar (Veterinærinstituttet, Norway), and incubated at 25 ± 0.25^oC and 18 ± 0.25^oC.

Identification of fungi

Macrofungi can often be identified to a species level by examining the fruiting bodies, but since all the fungi were retrieved as mycelium, this method of identification was not accessible. Therefore, the fungi were identified by sequencing.

DNA extraction

The AL protocol

The initially used protocol will be referred to as the AL protocol [52]. Genomic DNA was isolated from G.l.-1 and F.p.-5 mycelium growing on MEA by transferring a mycelium piece of about 0.5cm^2 to 1.5 ml microtubes containing 350 µL lysis buffer (AL buffer, QIAGEN®) and a sterile steel bead (ø = 4 mm). The content of the tubes, including a blank tube containing 50 µL MilliQ water instead of mycelium, were homogenized in a RESCH® mixer mill for 3 minutes at 25 Hz. The tubes were spun down to compress the foam, and 10 µL of 20mg/ml Proteinase K (Sigma- Aldrich) was added. The tubes were then quickly vortexed and incubated on a heating block at 56 °C for 30 minutes, and then centrifuged for 5 minutes as 12000 x g, with 200 µL of the resulting supernatant being transferred into new microtubes. 4 µL RNAse (Amresco®, 0675-1G) with a concentration of 100 mg/ml was added, and the microtubes were vortexed and spun down to mix and collect then liquid. The tubes were then incubated for 15 minutes at room temperature to allow the RNase to work. DNA extraction was continued using a QIAcube Connect (QIAGEN®) utilizing a QIAamp® DNA Mini Kit (QUIAGEN®). The settings were set to DNA, QIAamp DNA Mini Kit, then Tissue, then Standard. The reagents EtOH, lysis buffer AL, wash buffers AW1 and AW2, and elution buffer AE all supplied by Qiagen®.

A nanodrop One (Thermo Fisher Scientific) was utilized to investigate the quantity and quality of genomic DNA isolated. The genomic DNA isolates and blanks were also run through a 1.5% agarose gel containing GelRed, with 5 µL genomic DNA and blanks in the wells. The DNA was visualized under UV light (6X TriTrack DNA Loading Dye, Thermo Fisher Scientific).

The ATL protocol

The second method will be referred to as the ATL protocol, which was almost identical to the initial method, except that a larger 2 ml microtube was used, that the AL lysis buffer (QIAGEN®) was replaced with an ATL lysis buffer (QIAGEN®), and that the steel beads were replaced by three smaller 3 mm beads. The rest of the protocol was identical to the AL protocol, except that no RNase was used. The goal with the ATL protocol was to use an SDS containing lysis buffer and utilize several smaller beads to increase the mechanical strain on the mycelium.

The heat shock (HS) protocol

The third method was based on heat shock and will therefore be referred to as the HS protocol. In this method, the mycelium was added to 2 ml microtubes containing 350 µL ATL lysis buffer (QIAGEN®). The microtubules were stored in a -80°C freezer for five minutes, before being transferred to an incubator (Eppendorf ThermoMixer® C) at 75°C for five minutes. The freezing and heating step was repeated a total of five times, before three sterile 3mm steel beads were added, and the samples underwent homogenization in a RESCH® mixer mill for 3 minutes at 25 Hz five times. The rest of the protocol follows the AL protocol, except that no RNase was added.

The dry freeze protocol

The fourth approach was based on freezing of the mycelium and then exposing it to mechanical stress by homogenization, and will be referred to as the dry-freeze protocol. This protocol started by transferring mycelium to 2 ml tubes together with three 3 mm diameter sterile steel beads. The tubes were then stored for 10 minutes in a -80°C freezer, before being homogenized in a RESCH® mixer mill at 25 Hz for 3 minutes. This freezing and homogenization step was repeated a total of five times. Afterwards, 350 µl ATL lysis buffer (QIAGEN®) was added, vortexed, and then quickly spun down in a microcentrifuge to gather the content of the tube. The protocol then followed the rest of the AL protocol starting with the addition of protease, except that no RNase was added.

The MicC and Mic protocols

The final two methods were crude microwave methods and will be referred to as MicC and Mic protocol, depending on if the sample underwent cleanup in a QIAcube Connect (QIAGEN®) or not, respectively. Small 0.5cm^2 mycelium pieces and 200 µl purified water was added to 2 ml microtubes. The microtubes were then microwaved at 650W for 30 sec and then vortexed. The microwave and vortexing steps were repeated a total of two times, and the liquid was then spun down in a microcentrifuge. One set of samples were sent to be cleaned up using a QIAcube Connect (QIAGEN®) utilizing a QIAamp® DNA Mini Kit (QUIAGEN®), as done in the other protocols.

A blank extraction control was produced for every method by replacing the mycelium with 50 µl MilliQ water. All samples were run through a Nanodrop One (Thermo Fisher Scientific) to estimate the DNA concentration and to investigate purity. The genomic DNA isolates and extraction blanks were also run through a 1.5% agarose gel containing GelRed, with 5 µL genomic DNA and blanks in the wells. The DNA was visualized under UV light.

PCR Amplification of ITS, tef1α, and rpb2

Primer selection

Table 2. Primers used for the sequencing of genes utilized to identify the fungi.

GENE	PRIMER PAIR	FORWARDS SEQUENCE (5'-3')	REVERSE SEQUENCE (5'-3')	PRODUCER
ITS	ITS1/ITS4	TCCGTAGGTGAACCTGCGG	TCCTCCGCTTATTGATATGC	Invitrogen, Thermofisher scientific
TEF1A	EF1-1018F/ EF11620R	GAYTTCATCAAGAACATGA	GACGTTGAADCCRACRTTGTC	Biosearch technologies, Risskov Denmark
RPB2	bRPB2-6F/ bRPB2-7.1R	TGGGGYATGGTNTGYCCYGC	CCCATRGCYTGYTTMCCCATGDC	Eurofins genomics, Ebersberg Germany

The goal was to identify the two cultivated fungi G.l.-1 and F.p.-5 by sequencing. The chosen genes were ITS*, tef1α,* and *rpb2,* with specific primers listed in *Table 2* The ITS was chosen as the universal barcode

for fungi, and the primers ITS1 and ITS4, retrieved from [53], were selected as the forwards and reverse primers.

Unfortunately, *its* is by itself not enough to determine the fungi to the species level, which is why *tef1α* and *rpb2* were used as secondary barcodes to provide a higher resolution. The *tef*1α primers was selected based on Stielow *et al*. [2015] [54], and chosen due to their high PCR success rate in Basidiomycota, with the primers for the gene, EF1-1018F and EF1620R retrieved from the same paper. The *tef*1α primers were ordered from Biosearch technologies, Risskov Denmark.

The *rpb*2 primers bROB2-6F and bRPB2-7.1R were chosen based on Kim *et al.* [2007] [55] and Cabbario *et al.* [2019] [56], and retrieved from Matheny *et al.* [2005] [57]. The *rpb2* primers were ordered from Eurofins genomics, Ebersberg, Germany.

PCR amplification

The primers were mixed with MilliQ water to achieve a 5 pmol/µl concentration. The master mix for *its* amplification were composed of 17 µl MilliQ water, 3 µl 5 pmol/µl ITS1 primer, and 3 µl 5 mol/µl ITS4 primer per sample. The master mixes for *tef1α* and *rpb2* amplification were composed of 18 µl MilliQ water, 1.5 µl 5 pmol/µl forwards primer, and 1.5 µl 5 pmol /µl reverse primer per sample.

Master mixes were added to PCR tubes with PuReTaq™ Ready-To-Go™ PCR beads in a plate (407513-PLT), with 23 µL master mix and 2 µL DNA in the case of *its,* and 21 µL master mix and 4 µL DNA in the cases of *tef1α* and *rpb2.*

The first attempt at PCR was performed in a Veriti 96 well thermal cycler (Applied biosystems, Thermofisher Scientific) The PCR cycling conditions for ITS, retrieved from Liu *et al.* [2021][37], was as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles at 95°C for 45 sec, 54°C for 45 sec and 72°C for 90 sec and a final extension of 72°C for 6 min. The samples were held at 4°C.

The PCR cycling conditions for *tef*1α, retrieved from Hoang *et al.* [2019] [58], was as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 50 sec, 48°C for 50 sec and 72 °C for 50 sec and a final extension of 72 °C for 7 min. The samples were held at 4°C.

The PCR cycling conditions for *rpb*2, retrieved from Kim *et al.* [2007] [55], was as follows: initial denaturation at 95°C for 10 min, followed by 39 cycles at 95°C for 1 min, 55°C for 1 min +3 sec / cycle and 72 °C for 1 min and a final extension of 72 °C for 10 min.

All the protocols were modified to optimize the conditions of the PuReTaq™ Ready-To-Go™ polymerase. The amplification products were visualized under UV on a 1.5% agarose gel after electrophoresis.

Additional PCR reactions with annealing temperature gradients were used. The PCR protocol for amplification of *tef1α* in the F.p.-5 samples was as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 50 sec, annealing at 48, 49, 50, 51, 52 and 53°C for 50 sec and 72 °C for 50 sec and a final extension of 72 °C for 7 min. This PCR was performed in a Veriti 96 well thermal cycler.

The *rpb2* PCR protocol used included the 8-step gradient amplification as follows: initial denaturation at 95°C for 10 min, followed by 35 cycles at 95°C for 1 min, annealing at 55, 55.7, 56.9, 58.8, 61.1, 63, 64.3, and 65°C for 1 min +3 sec / cycle and 72 °C for 1 min and a final extension of 72 °C for 10 min. PCR was performed in a Bio-Rad T100 Thermal Cycler.

The temperature gradient PCR products were visualized under UV on a 1.5% agarose gel.

A final PCR protocol was performed to amplify *tef1α* from F.p.-5 by touchdown PCR.The PCR protocol was as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 50 sec, annealing at 48, 49, 50, 51, 52 and 53°C for 5 sec and 72 °C for 50 sec and a final extension of 72 °C for 7 min.

The PCR products were sent to Eurofins genomics (Ebersberg, Germany) for sequencing.

Analysis of sequences

To prepare for phylogenetic analysis, reference sequences for ITS, *rpb2*, and *tef*1α loci of *Ganoderma* and *Fomitopsis* were retrieved from GenBank, with the reference sequences selected based on recent literature [59] [37] .

The forwards and reverse Sanger sequenced DNA sequences of the two fungi were analyzed in Geneious Prime® Build 2023-04-27 (Biomatters Inc.), with low quality ends trimmed and low-quality reads inside the sequences edited manually based on quality and consensus suggestions.

Multiple sequence alignments were performed for each locus independently utilizing the MAFFT v7.490 plugin in Geneious Prime® Build 2023-04-27[60]. The ITS, *tef1α,* and *rpb2* alignments were concatenated.

Following phylogenetic analyses were performed with the CIPRES Science Gateway[61]. The concatenated datablocks were analyzed by ModelTest-NG (0.1.7) on XSEDE to determine[62]. The determined models and the concatenated data blocks were used for phylogenetic interference by maximum likelihood and Bayesian interference utilizing RAxML-NG (1.20)[63] and MrBayes on XSEDE (3.2.7a)[64] respectively.

Material production approaches

Material production approach: Liquid 1- Petri dishes

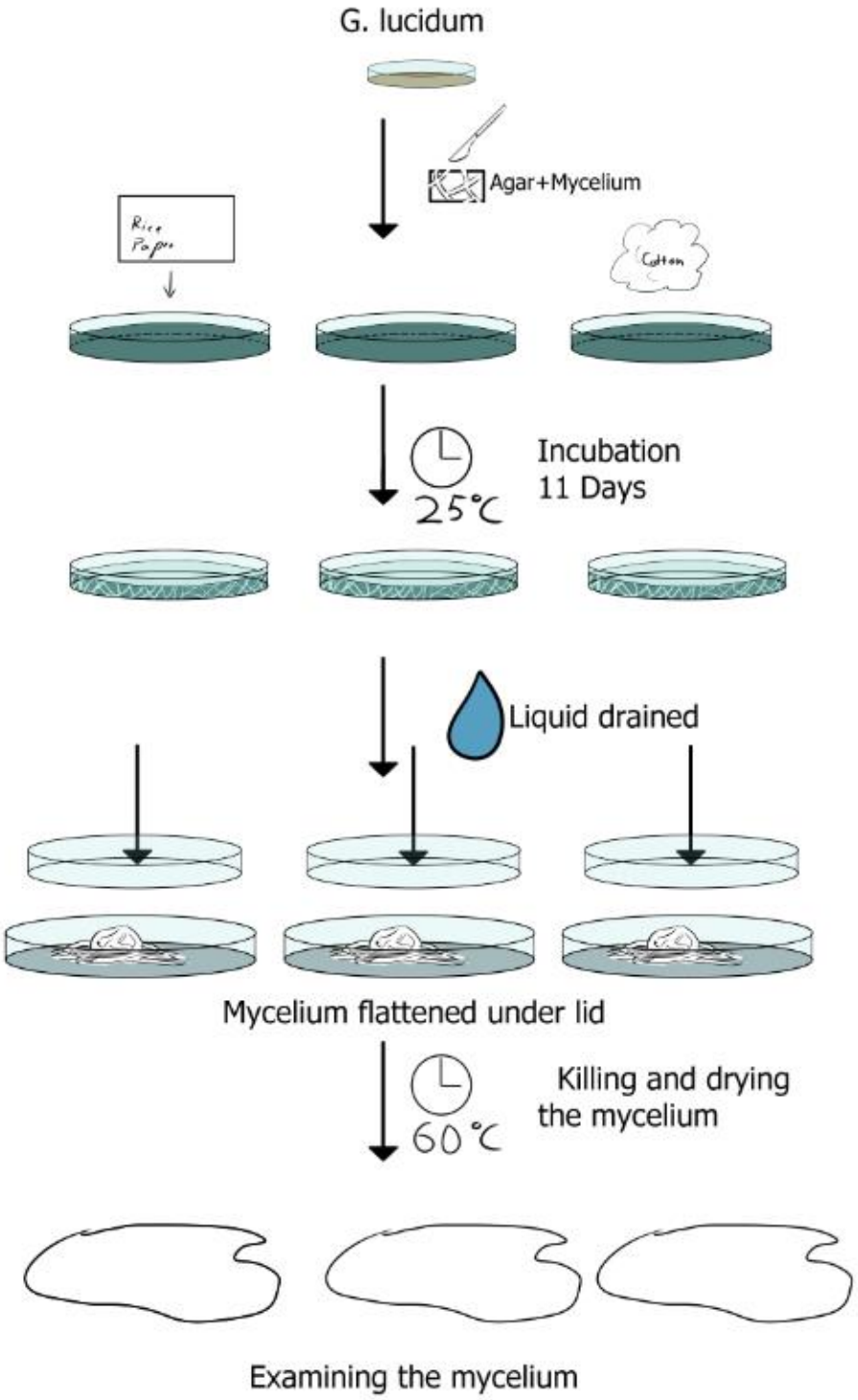

Figure 14 Visual representation of the material experiment, based on mycelium grown in liquid media, and with and without the addition of rice paper and cotton wool.

The approach to the first attempt of producing mycelium-based material utilizing liquid medium is visualized in *Figure 14*. In preparation, rice paper (Coop Rispapir 200g, EXOTIC FOOD PUBLIC COMPANY LTD) and cotton wool (Coop Bomull 100g, LEMOINE FRANCE) were sterilized by autoclave at 121°C for 20 minutes and by UV exposure for 30 minutes. The rice paper melted during the autoclave but could be scraped out from the glass container as fragments. Petri dishes containing 30 ml Malt extract broth (MEB), 30 ml MEB and UV or autoclave treated cotton wool, or 30 ml MEB and UV or autoclave treated rice paper were inoculated by pieces of agar covered by G.l.-1 mycelium. The petri dishes were initially incubated in a New Brunswick™ Innova® 42R/Ref shaker (Eppendorf, USA) at 25 ± 0.25°C for 8 days. At this point, liquid had almost entirely disappeared, so 5 ml of MEB was added to the plates, and the incubation continued for 3 more days. The liquid was then drained from the petri dishes, leaving mycelium and what it had bound left in the dishes. The mycelium was then transferred to the lid of the petri dish, while the bottom of the petri dish was put on top of the mycelium to flatten it by light pressure. The dishes were transferred to the same incubator, now at 60°C to kill and dry the fungi, with the pressure to flatten the mycelium maintained with the assistance of tape. After 48 hours, the petri dishes were removed from the incubator.

Material production approach: Liquid 2- Incubation construct

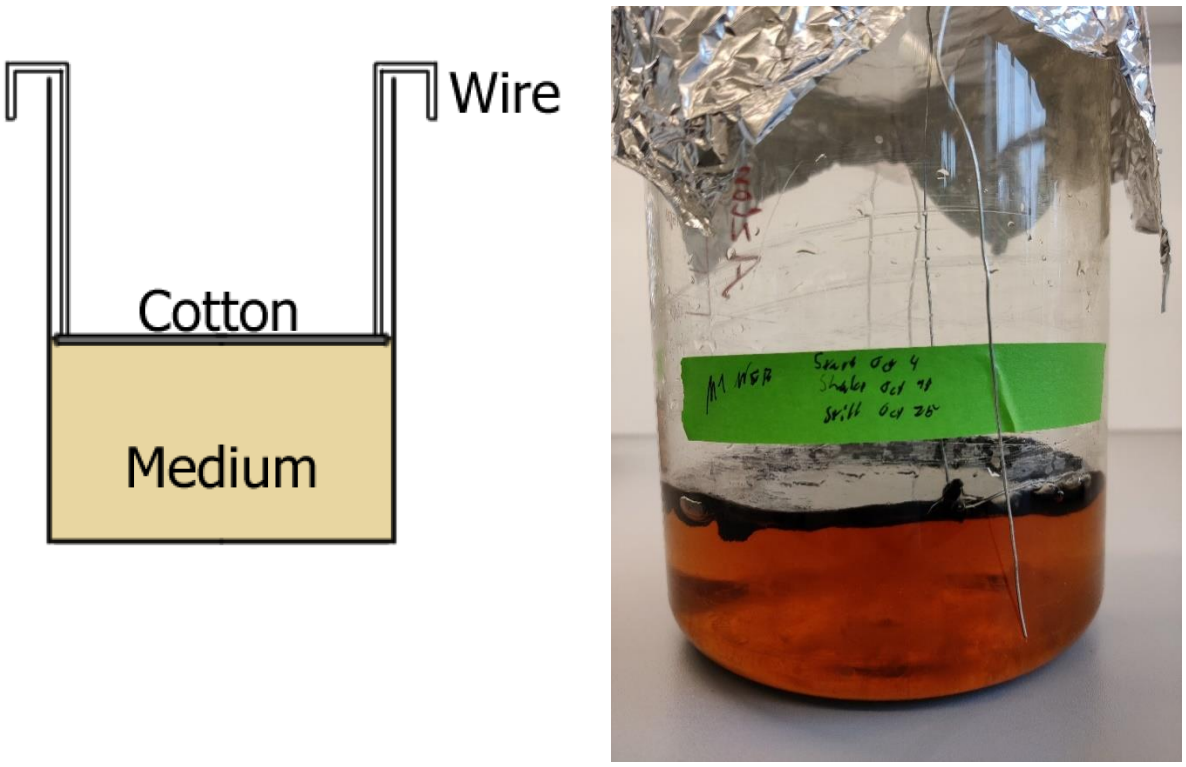

Figure 15. Illustration (left) and picture (right) of incubation chamber construct containing a cotton T-shirt piece (black) upheld by steel wire and covered with aluminum foil.

First, two incubation chambers, illustrated in Figure 15. , were constructed. The goals were to provide the mycelium material to grow through, and to ensure constant access to MEB, while still maintaining sterile conditions. To do this, a portion of an old cotton T-shirt was suspended stretched out in a circle of steel wire, folded on the edges, and sewn together. The T-shirt was then transferred to a 2 L glass

beaker. The steel wires were threaded through an aluminium foil covering the beaker, and the holes produced by the thread passing through were taped to ensure sterile conditions. The incubation chamber was sterilized in an autoclave at 121°C for 20 minutes.

Improving evenness started by producing liquid inoculum. To do this, 100 ml of MEB in Erlenmeyer flasks were inoculated with G.l.-1 or F.p.-5 mycelium and incubated for a week at 25 ± 0.25°C in a New Brunswick™ Innova® 42R/Ref shaker (Eppendorf, USA)**.**

To begin incubation, around 300 ml of MEB and 30 ml inoculum was added to the incubation chamber construct under sterile conditions. The construct was then transferred to New Brunswick™ Innova® 42R/Ref shaker (Eppendorf, USA), which was set to 25 ± 0.25°C and 60 RPM for two weeks, and one week at 25 ± 0.25°C without shaking.

Figure 16. A Norwegian "syltepresse" used to flatten the mycelium.

After the incubation, the mycelium covered T-shirt was placed in a wooden press, shown in *Figure 16*, between two pieces of wrapping plastic. The screws were tightened, and the press containing the mycelium was placed within the New Brunswick™ Innova® 42R/Ref shaker (Eppendorf, USA) set to 60± 0.25°C overnight. The screws were then loosened and left in the incubator for another night at 60 ± 0.25°C.

In the final step, the mycelium covered T-shirt was cut in two, and one half was placed in 10% glycerol solution (Glycerol 86%, VWR chemicals 20D234128, diluted in tap water) overnight to improve flexibility. The mycelium pieces were finally dried at room temperature and examined.

Material production approach: Liquid 3 - Boxes

Boxes (Boks SMARTSTORE CLASSIC 2 2L m/lokk, 17x21x11 cm, Jysk, Norway) containing 10 x 12 cm nylon tulle (Rainbow Tekstil, Oslo) pieces were sterilized in an autoclave at 121°C for 20 minutes. Around 750 ml MEB was then added to the boxes under sterile conditions, and they were inoculated with around 75 ml G.l.-1 and F.p.-5 liquid inoculum. The boxes were stored in a New Brunswick™ Innova® 42R/Ref shaker (Eppendorf, USA) set to 25°C, 60 ± 0.25 RPM for 19 days.

The resulting mycelium was removed from the boxes, with some loose brownish mycelium falling off. The dense white mycelium was placed in the wooden press between two sheets of wrapping plastic, the screws tightened, and the press was placed into the New Brunswick™ Innova® 42R/Ref shaker (Eppendorf, USA). The incubator was set to 60 ± 0.25°C overnight. The screws were then loosened and placed into the incubator for another night.

The flattened and dried mycelium pieces were placed in a 10% glycerol bath (Glycerol 86%, VWR chemicals 20D234128, diluted in tap water) overnight. The 10% glycerol bath did not grant the desired results, so one sheet of mycelium was cut up into 4x4 cm pieces, and the pieces put into 0%, 10%, 25%, 50%, and 86% glycerol baths overnight. The mycelium pieces were finally dried at room temperature and examined.

Material production approach: Liquid 4 - Flipping, folding, and covering.

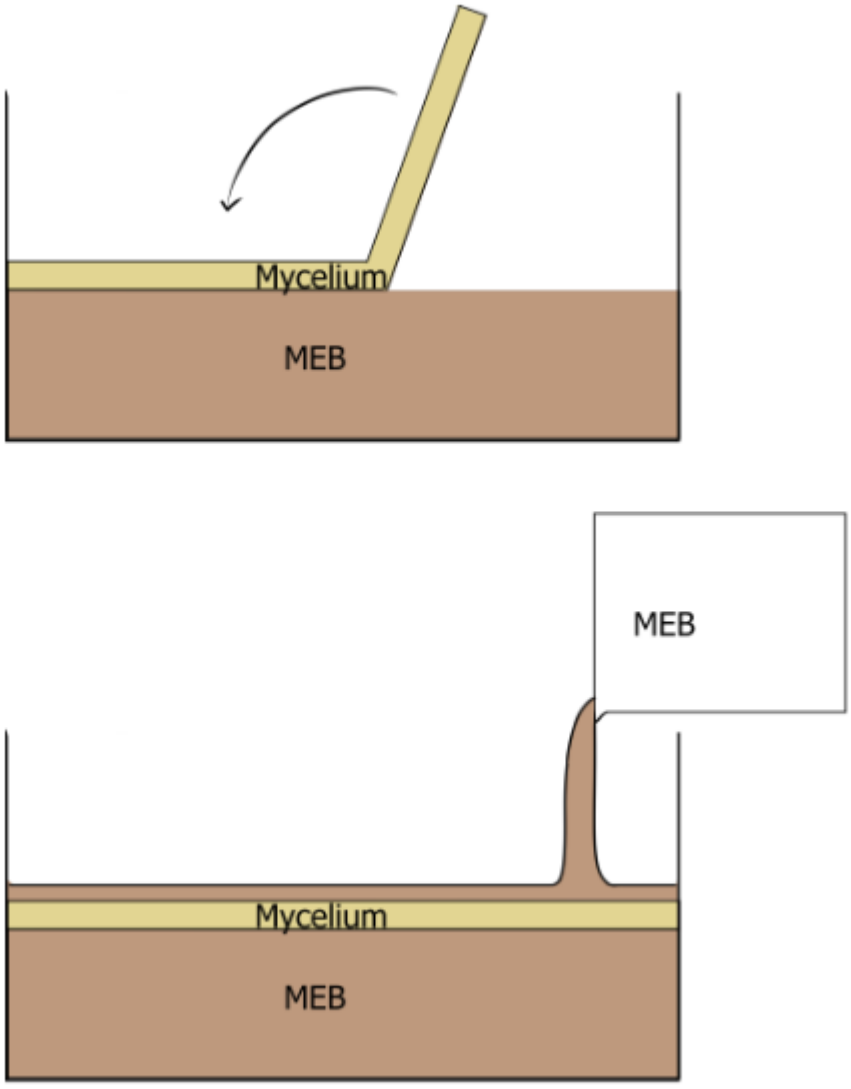

Figure 17 The methods used to produce layered mycelium. The upper picture describes how the mycelium was folded onto itself, while the lower picture describes how the mycelium was covered with a new layer of MEB.

Two folding and covering approaches were done in parallel. Boxes (17x21x11 cm) with 375 ml MEB were inoculated with 37.5 ml G.l.-1 liquid inoculum and incubated in a New Brunswick™ Innova® 42R/Ref shaker (Eppendorf, USA) at 25° ± 0.25°C at 60 RPM for 6 days, then at 25 ± 0.25°C without shaking for 27 days. One box had the mycelium covered with 5 mm of MEB, while the second box had the mycelium folded onto itself, as seen in *Figure 17*. The boxes were then incubated for another 22 days in the shaker at 25 ± 0.25°C, no shaking. The resulting mycelium sheets underwent the same press and heat treatment in previous liquid attempts, and the dried mycelium sheets were initially covered by 25% glycerol for 3 days, then 50% for one day, and dried in room temperature.

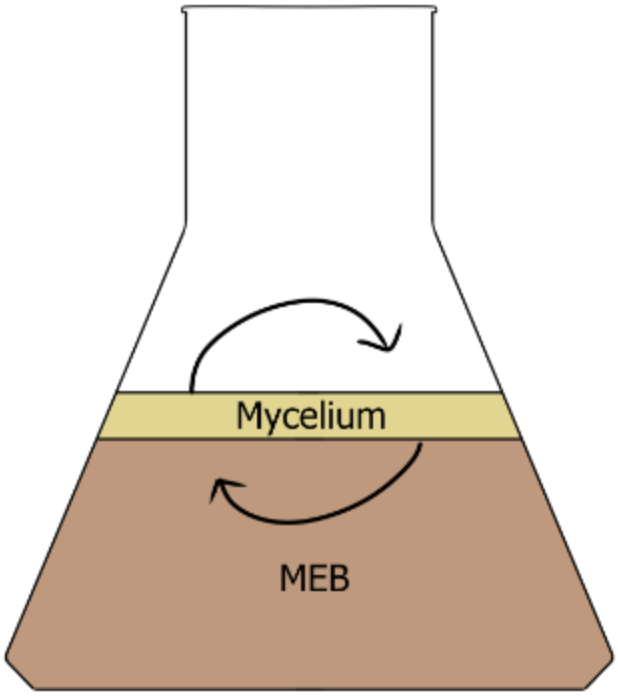

Figure 18 Illustation of how the mycelium was flipped in the Erlenmeyer flask, exposing the underside of the mycelium to the air.

As a flipping experiment, two Erlenmeyer flasks containing 250 ml MEB and 25 ml G.l.-1 and F.p.-5 were incubated at room temperature without shaking for 27 days. The mycelium sheet on the surface was then flipped using an inoculum loop and incubated for 7 days, as seen in in *Figure 18*. This flipping and incubation was repeated a total of two times. The resulting mycelium sheets were placed in the press and underwent the same drying treatment as previous liquid approaches and was then placed in a 50% glycerol bath overnight. The mycelium pieces were finally dried at room temperature and examined.

Material production approach: Liquid 5: Bioreactor

An attempt was made to utilize a bioreactor to produce mycelium. Growth conditions was retrieved from "Optimisation of biomass, exopolysaccharide and intracellular polysaccharide production from the mycelium of an identified *Ganoderma lucidum* strain QRS 5120 using response surface methodology" [65].

A Infors HT minifors bioreactor containing 1.4 L 1 g/L yeast extract (Merck VM846853823), 1 g/L KH_2PO_4 (Merck AM1089873715), 0.5 g/L $MgSO_4$ (VWR chemicals 14G090022), 4 g/L NH_4Cl_2 (Sigma Aldrich AM1764645138) 26.5 g/L glucose (VWR Chemicals, 22C284126) at pH 4 with an aeration rate of Q_g= 1 air/L and stirring at 100 rpm was inoculated with 100 mL inoculum produced by inoculating MEB with G.l.-1, and incubating it in a New Brunswick™ Innova® 42R/Ref shaker (Eppendorf, USA) at 25 ± 0.25°C at 60 RPM for 1 week, and 120 RPM for 1 day. Problems with the pH reading in the bioreactor incubation meant that the pH had to be adjusted manually in the beginning, but automatic pH adjustment using 5M NaOH and 5M H_2SO_4 was possible starting the second day. The bioreactor ran for two weeks. The mycelium was collected using a sieve, placed in the wooden press between two sheets of wrapping plastic, and dried at 60°C for 2 days.

Material production approach: Solid state cultivation

An attempt was also made to produce a composite material composed of saw dust and mycelium. First, spawn was produced. This was done by submerging 1 L rye (Gartnerbutikken, Norway) in water for 48 h and distributing it evenly into two small mycobags (Unicorn Mycobag 0,5 µM filter - 4a, Gartnerbutikken, Norway), and heat sealing the bags. The mycobags were sterilized in an autoclave at 121°C for 20 minutes. Around 20 pieces of G.l.-1 and F.p.-5 mycelium from agar plates were then transferred to the bags under sterile conditions, the bags resealed, and mixed by pressing on the bags, moving the rye around. The bags were stored at room temperature in a waterproof box and sprayed with water daily. Water accumulating in the bottom of the box was removed when deemed too much. The spawn was mixed every few days to ensure more even growth throughout the rye. The spawn was allowed to grow for 21 days.

The next step was preparing the sawdust to be inoculated. Oak and spruce sawdust was submerged in water for 48 hours, before being drained utilizing a sieve. About 4 L of sawdust was distributed into large mycobags (Unicorn Mycobag 5 µM filter - 3b, Gartnerbutikken, Norway). The bags were heat sealed and autoclaved at 121°C for 20 minutes. When the spawn was ready, the spawn was mixed into the mycobags with a ratio of 20:80 w/w spawn to sawdust under sterile conditions. The mycobags were heat sealed and mixed by pressing on the side of the bags. The bags were stored in boxes and sprayed with water and mixed daily for 18 days. The mycelium sawdust mix was then transferred to autoclaved 2 L boxes (Boks SMARTSTORE CLASSIC 2 2L m/lokk, Jysk, Norway), and incubated at room temperature for 15 days. The boxes were then examined, and then placed in a New Brunswick™ Innova® 42R/Ref shaker (Eppendorf, USA) set to 60°C for 3 days.

Results

Cultivation of mycelium

Of the five fungal isolates retrieved, only G.l.-1 and F.p.-5 grew on the MEA plates. Within three days of inoculation of new MEA, white powder mycelium covered half the plate, and continued to grow until the entire plate was covered. In culture tubes, a layer of dense white mycelium on the top of the liquid was formed within two weeks, while the upper parts of the liquid contained loose hypha. F.p.-5, developed a white, loose, and layered mycelium covering the entire surface. In MEB with two weeks incubation, the fungus produced a thick but loose layer of fungi on the surface of the broth, with loose hypha present in the liquid. The other three fungal isolates were also inoculated on alternative media, but no growth was observed.

Identification of the fungi

DNA extraction

Table 3 Nanodrop measurements of the DNA extracts and blanks produced during the first extraction attempt.

Sample	Concentration (ng / µl)	A260/A280	A260/A230
G.l.-1	2.7	1.90	0.14
F.p.-5	120	1.57	0.54
Blank Control	2.7	1.60	0.14

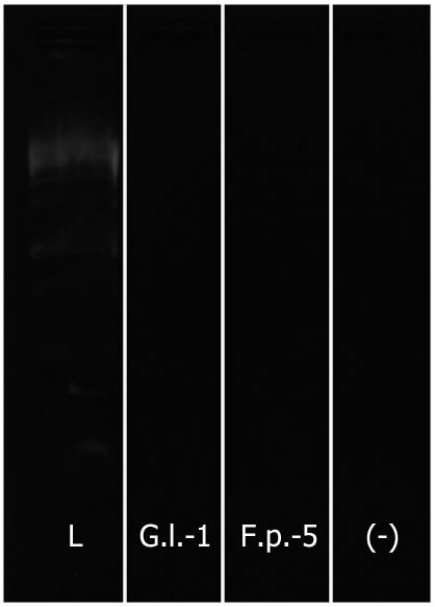

Figure 19 Electrophoresis of the first DNA extraction products. L represents the 1 kb DNA ladder. G.l.-1 is the G.l.-1 isolate, F.p.-5 is the F.p.-5 isolate, (-) is the extraction blank control.

The first DNA extraction attempt failed. The Nanodrop results, seen in *Table 3* were already suspect, especially with the blank control providing almost the same concentration and absorption measurements as G.l.-1, while F.p.-5 had a supposedly large DNA concentration. The absence of DNA in all samples was confirmed by gel electrophoresis, as seen in *Figure 19*.

A series of harsher DNA extraction protocols, including ATL, heat shock, dry-freeze and microwave with and without cleaning was performed to attempt DNA extraction. All samples, except the microwave samples which weren't cleaned, were examined using Nanodrop. The ideal values were more than 10 ng/µl DNA, a A260/A280 ratio around 1.8 and a A260/A280 ratio around 2, but lower A260/A280 ratio was expected due to the QIAamp® DNA Mini elution buffer being salt based.

Table 4 The Nanodrop measurements of the samples produced by the alternative extraction protocols.

Sample	Extraction	Concentration (ng / µl)	A260/A280	A260/A230
G.l.-1	ATL	36.0	1.94	0.81
F.p.-5		50.2	2.12	1.27
Blank control		2.4	3.17	0.12
G.l.-1	Dry-freeze	43.0	2.12	1.13
F.p.-5		65.9	1.97	1.09
Blank control		3.2	2.32	0.16
G.l.-1	HS	15.9	2.10	0.55
F.p.-5		41.9	2.11	1.17
Blank control		2.3	2.61	0.13
G.l.-1	MicC	11.4	2.00	0.43
F.p.-5		6.2	1.95	0.32
Blank control		8.0	1.73	0.31
G.l.-1	Mic	No nanodrop performed		
F.p.-5				

Blank control		

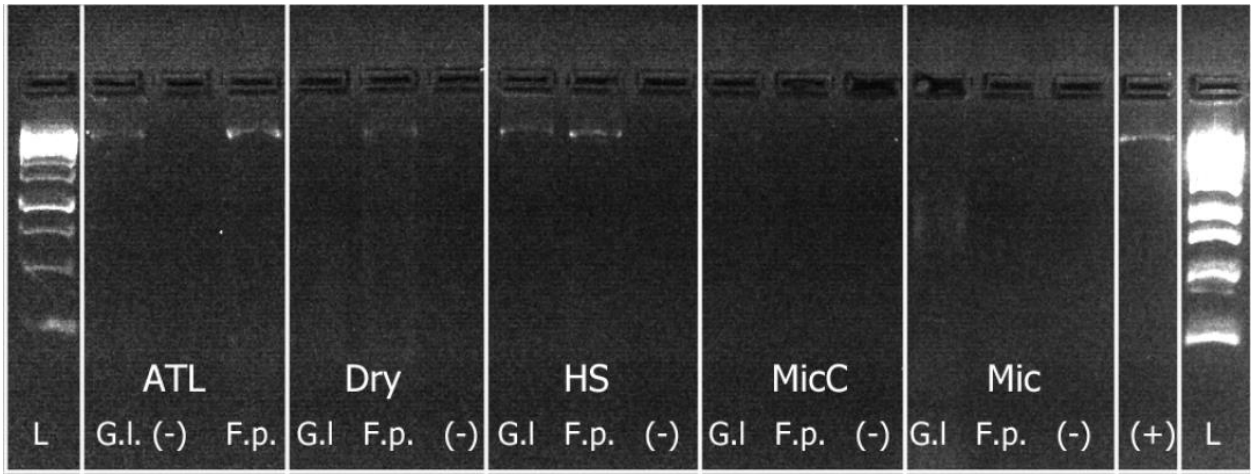

Figure 20 Gel with samples from the alternative extraction protocols under UV. ATL, Dry, HS, MicC and Mic indicates which protocol was used to produce the samples. L indicates 1kb DNA ladder, G.I.-1 is the G.I.-1 isolate, F.p.-5 is the F.p.-5 isolate. (-) indicates the extraction blank control, while (+) indicates a positiv control.

The nanodrop results, seen in *Table 4* were already promising, with all mycelia containing samples having high DNA concentrations and A260/A280 ratios of around 2. The A260/A230 ratios were low, but this was probably due to the DNA extraction technique. The blank extraction sample measurements were also slightly higher than expected, but this is probably due to the nanodrop being blanked with MilliQ water instead of AE elution buffer (Qiagen®).

The successful DNA extraction using the ATL protocol and the HS protocol, with some success with the dry-freeze protocol, indicated by the bands larger than 10 kb in *Figure 20*. The Mic protocol seems to only have produced a smear with G.I.-1, which is probably composed of cell components. The gel also confirmed that the F.p.-5 ATL sample and the ATL blank extraction sample at some point had been switched. The results were deemed good enough to continue with PCR amplification.

PCR

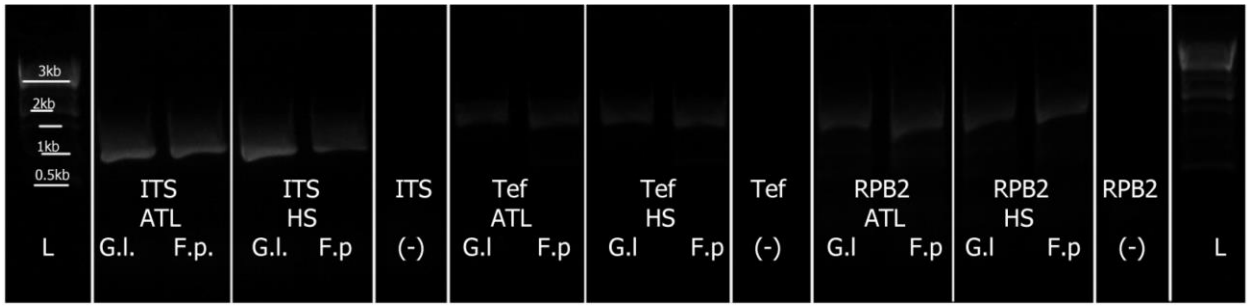

Figure 21 Gel under UV with the first PCR products. ITS indicates the use of ITS master mix, Tef the Tef1α master mix, and RPB2 the RPB2 master mix. ATL indicates that the DNA used was extracted using the ATL protocol, while the HS indicates DNA extraction using the HS protocol. G.I. is the G.I.-1 isolate, F.p. is the F.p.-5 isolate, (-) indicates negative control, while L indicates a 1kb DNA ladder.

The first amplification attempt, visualized on a gel in *Figure 21* succeeded in amplifying ITS from all the extractions, indicated by a strong band a bit under 1000 bases. Amplification of tef1α also succeeded, with a weaker, but still significant band of 1700 bases. Unfortunately, F.p.-5 seemed to have a secondary PCR product of around 800 bp. The rpb2 amplification produced significant bands of around 1700 bases, but all the samples also produced alternative PCR products, most around 1000 bases long. This indicated the need for alternative PCR protocols for tef1α amplification of F.p.-5 and for amplification of rpb2.

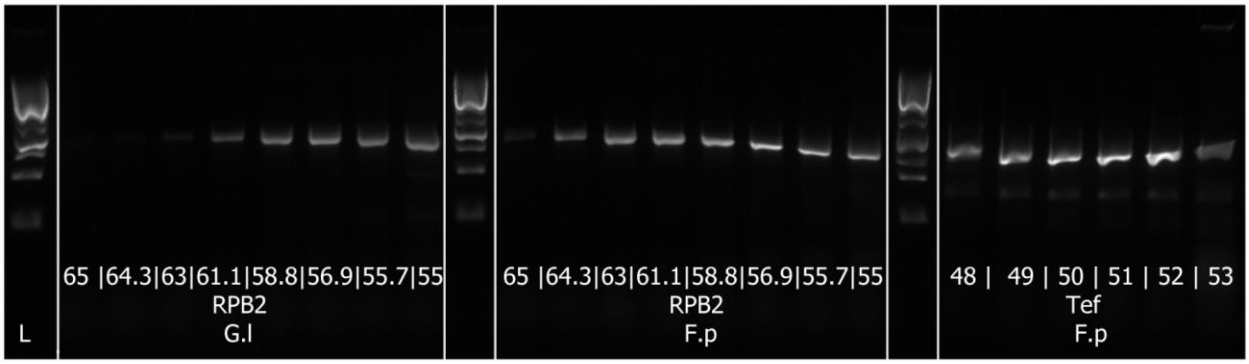

Figure 22 Gel under UV containing PCR products produced with a annealing gradient. The numbers indicate temperature in celsius. RPB2 and Tef indicate that the RPB2 and Tef1α master mixes were used to amplify the DNA respectively. L indicates the 1 kb DNA ladder, G.I. is the G.I.-1 isolate, F.p. is the F.p.-5 isolate.

The temperature gradient for the annealing step was implemented to produce PCR products with only a singular product, and therefore a singular band. In *Figure 22*, the results are seen. The gel seems to have shifted a bit, making determining size difficult, but the PCR bands for the RPB2 products seem to line up at around 1500 to 2000 base pairs. The Tef1α PCR products are around 1000 to 1500 base pairs, with second bands between 500 and 1000 bases.

Higher temperature produces lower amounts of product due to more rigoristic primer binding to the DNA, but also more specific PCR products. In the RPB2 samples, a secondary PCR product weas only produced at 55°C for G.I.-1, and at 55.7 and 55°C for F.p.-5. The samples produced at 56.9°C therefore had the required quality for DNA sequencing. The Tef1α PCR products were still not satisfactory due to producing secondary bands.

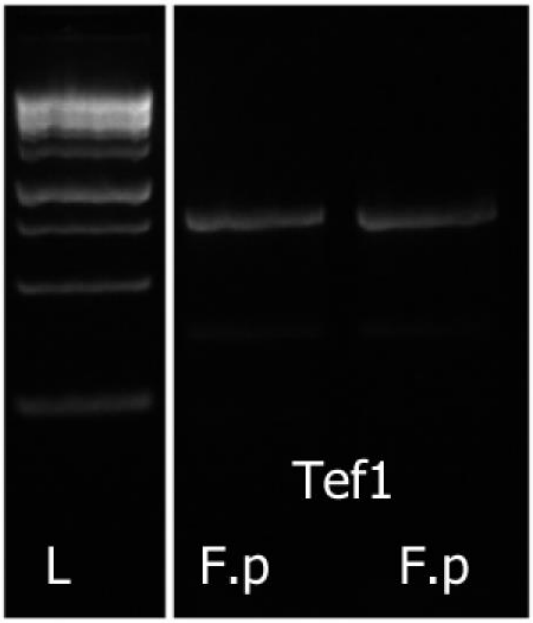

Figure 23 Gel under UV with the touchdown PCR products. L indicates ladder, Tef indicates that the samples were amplified using the Tef1α master mix, while F.p indicates that the DNA samples were extracted from F.p.-5.

In the end, touch-down PCR did not help in producing a PCR product with a single band in the gel when amplifying *tef1*α.

Analysis of sequences

High quality sequences of G.l.-1 ITS, *tef1*α, *rpb2,* and F.p.-5 ITS, and *rpb2* were retrieved from Eurofins. After building consensus sequences, the G.l.-1 ITS was 620 bp, the *tef1*α 684 bp, and the *rpb2* was 745 bp long. The F.p.-5 ITS sequence was 637 bp, and the *rpb2* was 783 base pairs long. The sequences were then aligned with the related sequences retrieved from the GenBank, and concatenated into data blocks, resulting in 117 sequences of 2089 bp in the *Ganoderma* dataset, and 74 sequences of 1178 bp in the *Fomitopsis* dataset.

The phylogenetic reconstruction was then performed. For the *Ganoderma* dataset, the best-fit substitution models based on maximum likelihood criterion were found to be: GTR for ITS; GTR and HKY for *tef1*α; and F81, HKY, and GTR for *rpb2*. The RAxML-NG analysis stopped automatically after 550 bootstrap replicates. For Bayesian information criterion, the best-fit substitution models were found to be: GTR for ITS; K2P, HKY, and GTR for *tef1*α. The Bayesian interference analysis was performed with two concurrent analyses of four chains run for 1000000 generations with the first 20% trees being eliminated as a burn-in.

The substitution models were also calculated for the *Fomitopsis* dataset. Based on the maximum likelihood criterion, the best substitution models were found to be: HKY for ITS; and HKY and GTR for *rpb2*. 850 bootstrap replicated were needed for the RAxML-NG to stop the analysis. For the Bayesian information criterion, the best substitution models were found to be: HKY for ITS; and GTR and SYM for *rpb2*. The Bayesian interference analysis was performed with two concurrent analyses of four chains for 1000000 generations with the first 20% trees being eliminated as a burn-in.

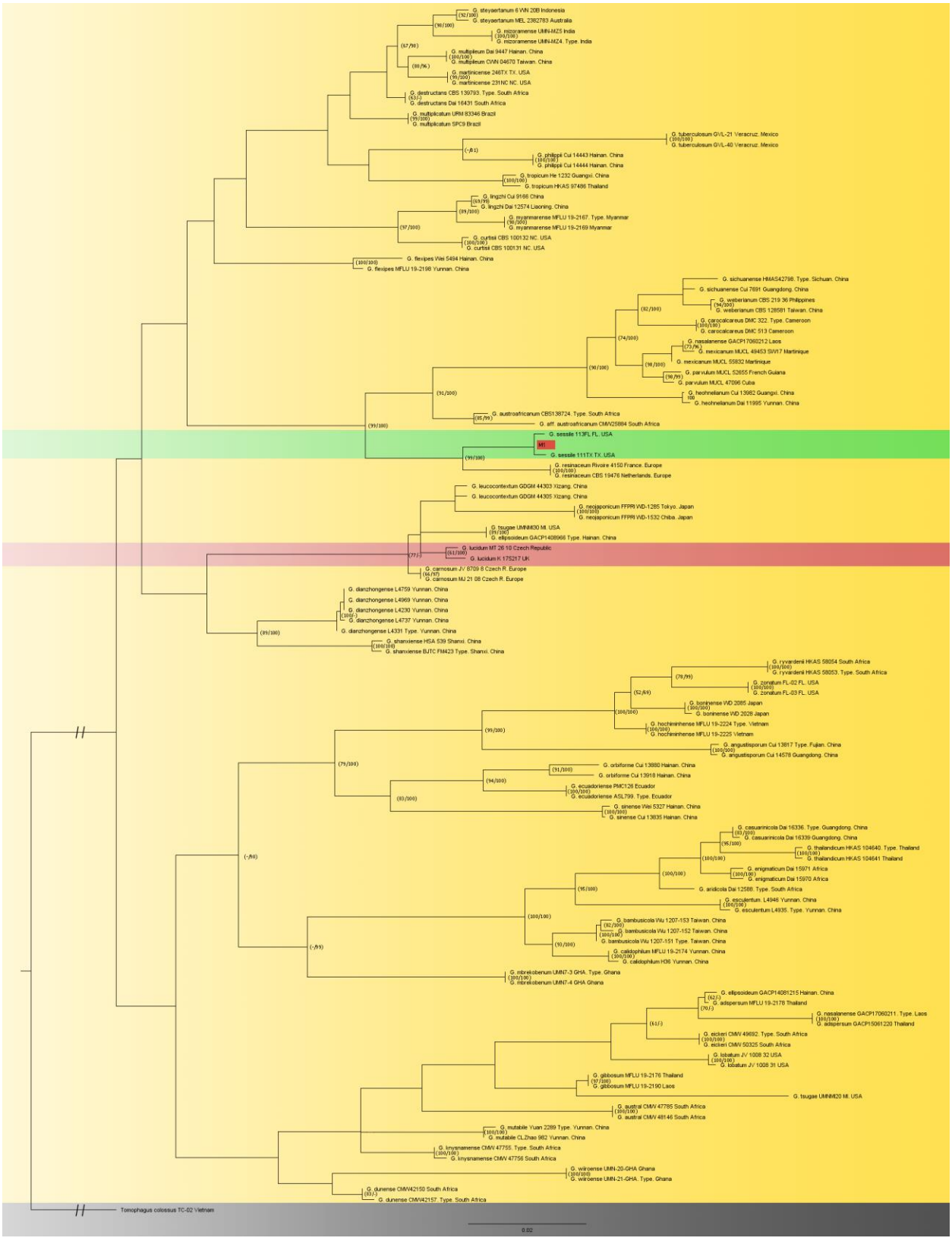

Figure 24 A consensus tree inferred from a multilocus dataset containing ITS, tef1α, and rpb2 sequences from Ganoderma species (coloured) with Tomophagus colossus(gray) as an outgroup. The tree was inferred by maximum likelihood, with the first

set of numbers at branches indicating bootstrap values over 60%, while the second indicates posterior probabilities over 80%. Two clades are also marked: a Ganoderma lucidum clade, marked in red, and a Ganoderma sessile clade marked in green. The intensely red marked M1 in the G. sessile clade is the G.l.-1 isolate. The scale bar represents the expected changes per site.

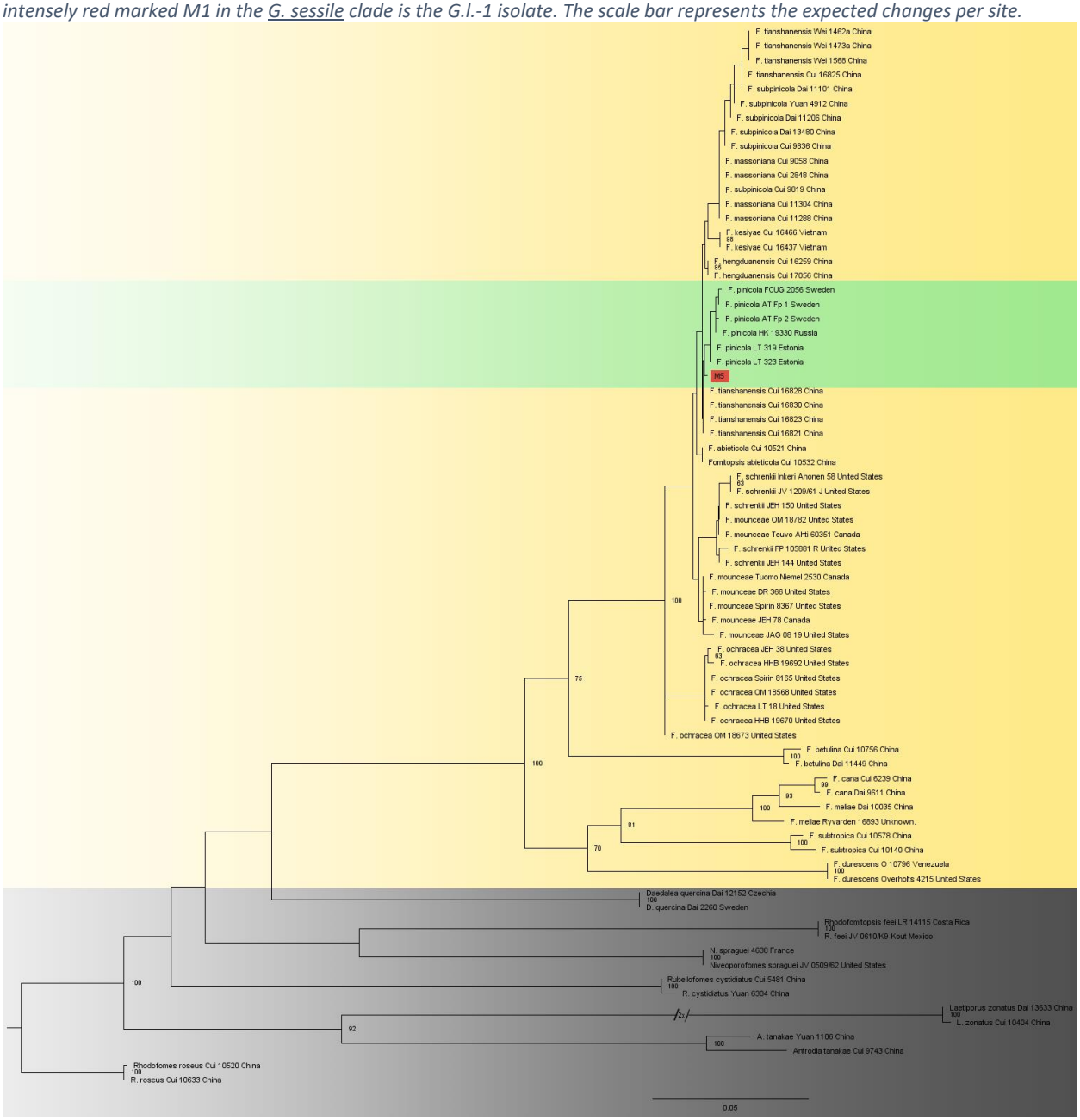

Figure 25 A consensus tree inferred from a multilocus dataset containing ITS, and rpb2 sequences from Fomitopsis species (coloured). Species belonging to genus are marked gray, with Rhodoformes roseus as an outgroup.The tree was inferred by maximum likelihood, with the numbers at branches indicating bootstrap values over 60%. The clade of F. pinicola is marked in green. The intensely red marked M5 refers to the *F.p.-5 isolate. The scale bar represents the expected changes per site.*

Satisfactory trees were interfered from the *Ganoderma* dataset using both maximum likelihood and Bayesian interference, seen in *Figure 24*. The tree placed the *G.l.-1* isolate, initially identified as a *G. lucidum* species, was placed in a well-supported *G. sessile* clade.

In the case of the *Fomitopsis* dataset, a satisfactory tree was only built using maximum likelihood, with the tree built using Bayesian interference being largely unresolved. The F.p.-5 isolate was placed within the *F. pinicola* clade.

Mycelium material production

Material production approach: Liquid 1- Petri dishes

Figure 26 Pictures of dried and pressed Ganoderma lucidum mycelium. Neg indicates that the mycelium was grown in only MEB. Cotton indicates mycelium grown in cotton wool and rice indicates mycelium grown on rice paper. Auto indicates that the substrate, being cotton or rice paper, was treated by autoclave, or by UV exposure I the case of UV.

The fungi grew nicely in the petri dishes after 11 days, with no sign of contamination, indicating that the treatment of the cotton wool and rice paper by autoclaving and UV was sufficient to prevent contamination. The presence of surfaces in form of the cotton and rice paper seemed to allow far faster growth of the mycelium than growth in only liquid media. After drying under pressure, the mycelium was examined (*Figure 26*). The mycelium pieces formed in the plates with only MEB or MEB and rice paper were less than 1 mm thick, and brittle, breaking apart with only light touch. The mycelium containing cotton wool was flexible, with a thickness of around 1 mm, but was easy to rip apart.

Material production approach: Liquid 2- Incubation construct

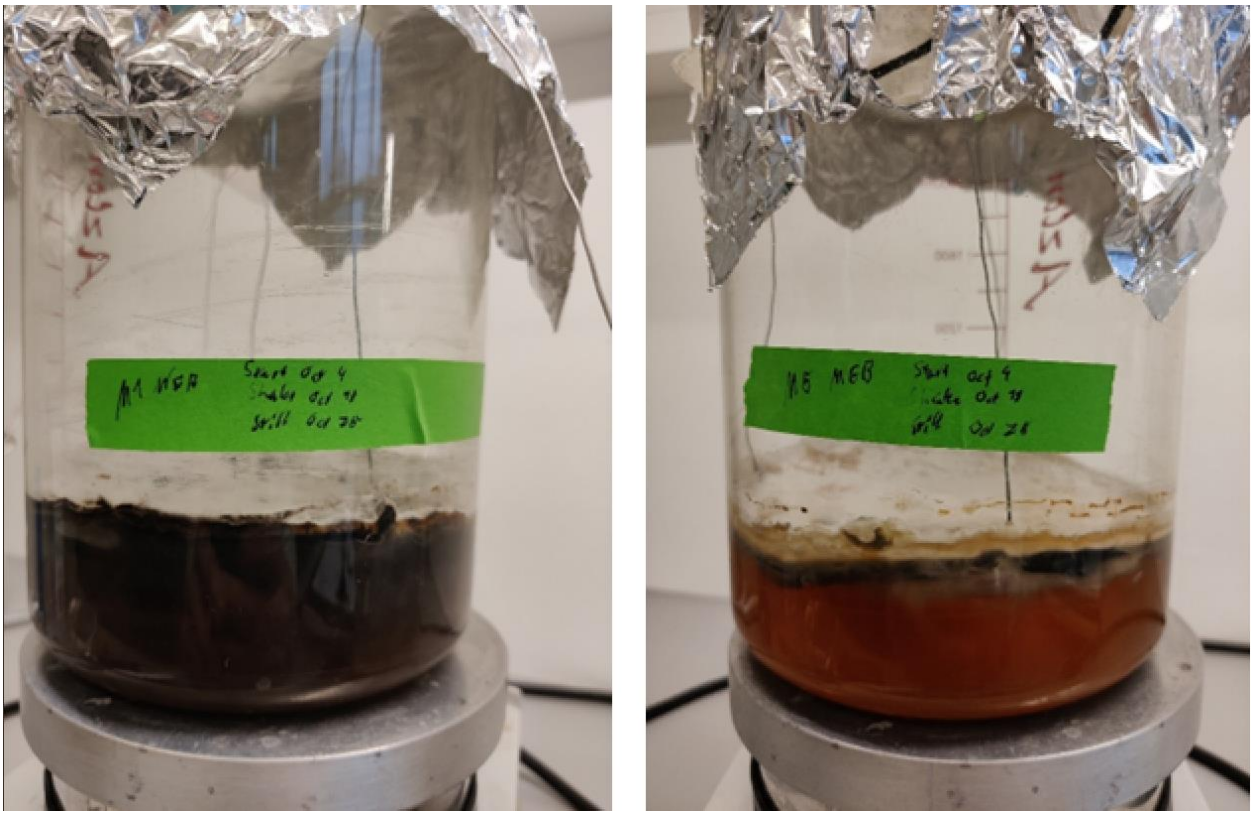

Figure 27. Two pictures of the two incubation chambers after incubation was completed. The left incubation chamber was inoculated with G.l.-1, while the right chamber was inoculated with F.p.-5.

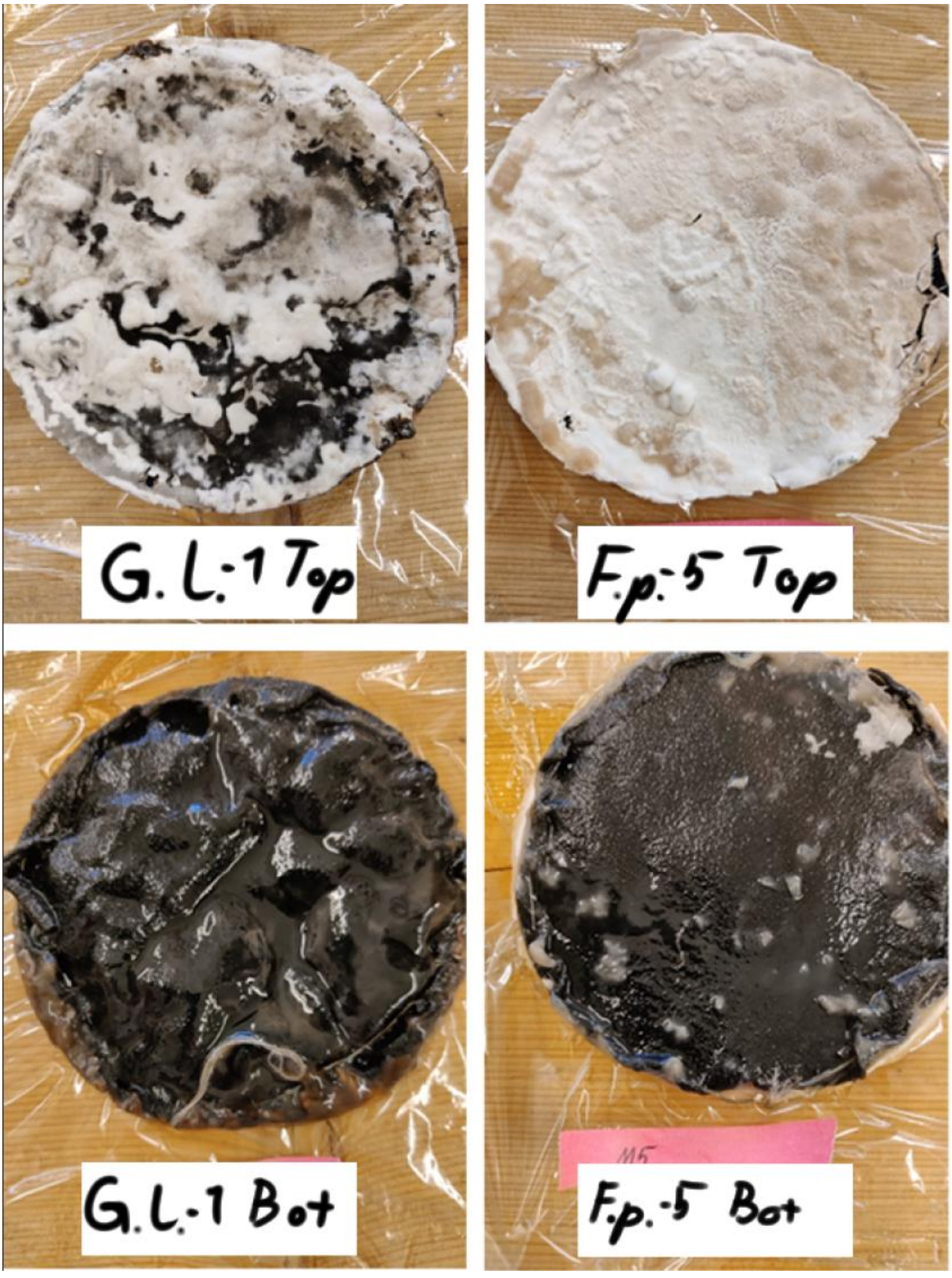

Figure 28. The pictures of the mycelium covered t-shirt material from the incubation chambers. Top indicates that the picture is from the side facing the air, while bot indicates that the picture is of the side facing the MEB.

As seen in *Figure 27.*, the G.l.-1 containing chamber contained a dark brown liquid with high amounts of mycelium in it. The top of the cotton T-shirt material was covered with mostly white, but also in some black mycelium. The liquid in the F.p.-5 containing growth chamber was a brighter more see through orange, more closely resembling the appearance of new MEB, while also containing significant amounts of mycelium. The cotton t-shirt material was extracted, with pictures of the cotton shown in *Figure 28.*. Examining the underside of the cotton revealed that the mycelium did not grow through the t-shirt, only around it. There was a significant layer of white mycelium on the top, which was flexible when wet. After the mycelium was dried, the G.l.-1 mycelium became brittle, while the F.p.-5 remained more flexible, but also had a powdery texture and tore easily. Treatment with glycerol made the G.l.-1 flexible once more and allowed it to be separated from the t-shirt as a less than 1 mm thick layer. The F.p.-5 mycelium seemed to shrink slightly in response to the glycerol treatment and did not become more flexible.

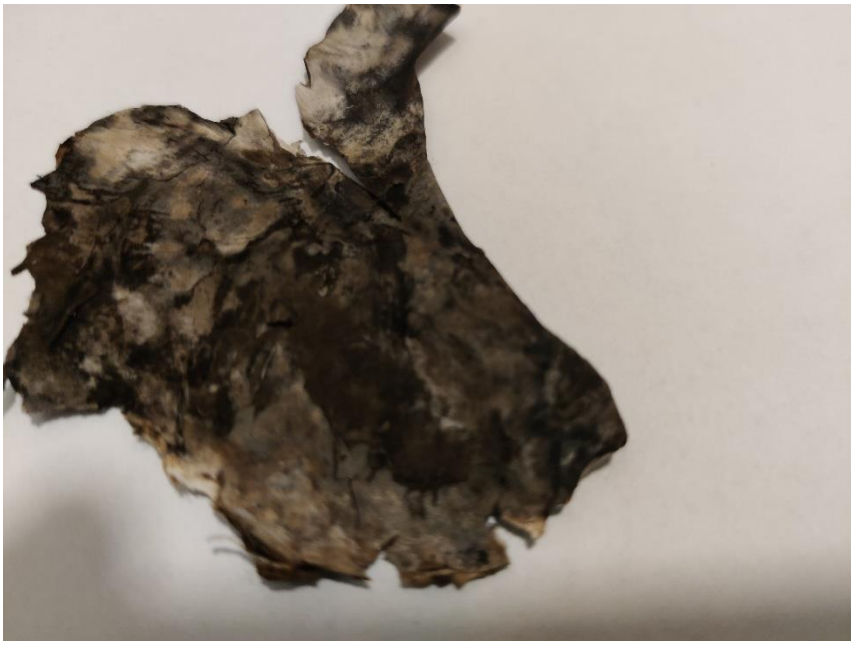

Figure 29 Picture of the G.l.-1 mycelium separated from the t-shirt.

One interesting aspect with the G.l.-1 was that it seemed to have taken on the colour of the t-shirt, becoming darkish brown to black, as seen in *Figure 29*, while the F.p.-5 seemed to have maintained its usual white to beige coloration. The material was still flexible 6 months after treatment.

Material production approach: Liquid 3- Boxes

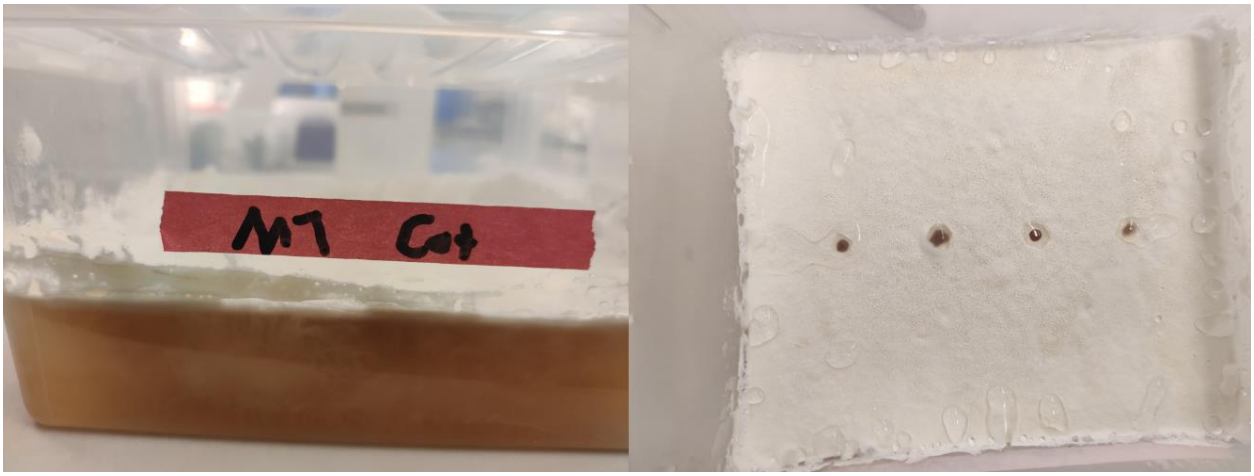

Figure 30 Picture of G.l.-1 grown in MEB on nylon tulle seen from the side (left) and seen from the top (right)

Figure 31 Picture of G.l.-1 grown in MEB on nylon tulle after being dried and treated with glycerol.

In *Figure 30* we see that the mycelium had grown in the MEB, producing loose brown mycelium structures in the liquid while producing a thin but more structurally sound layer of white mycelium on the surface. The mycelium was treated by heat under pressure, turning the sheet brittle. The mycelium sheet was then bathed in glycerol in an attempt to make it more flexible, but it did not work, as the sheet remained brittle when dried. The mycelium sheet was therefore cut up and treated with different concentrations of glycerol, with the result seen in *Figure 32*.

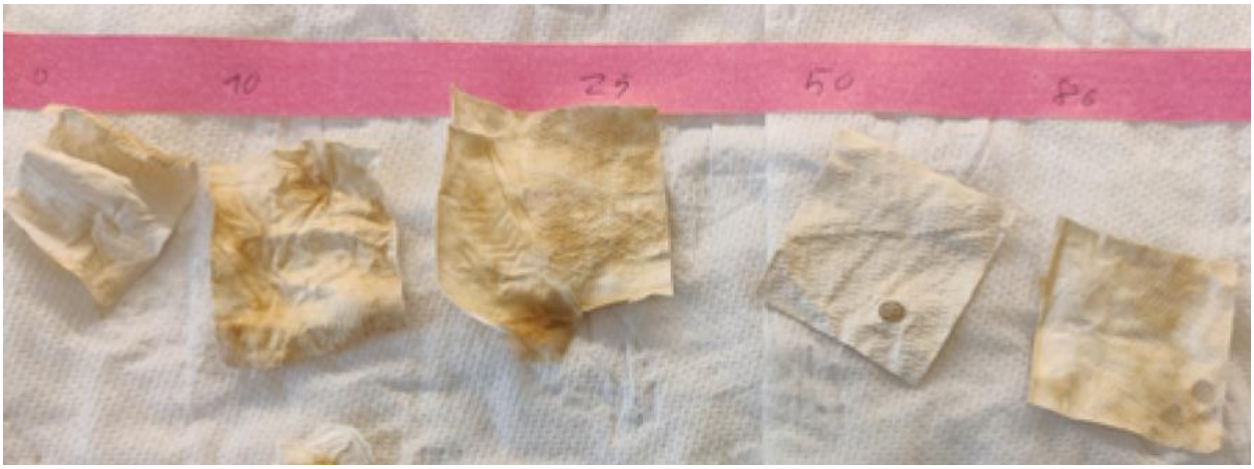

Figure 32 Picture of G.l.-1 grown in MEB on nylon tulle after being cut up and treated with differing concentrations of glycerol. The numbers on the tape represent the glycerol concentration in percentage.

The concentration of glycerol seemed to influence the degree of flexibility of the material to up to 50%, but after 50% the mycelium only took longer to dry and was sticky due to the glycerol. The flexibility was maintained for a few months, but slowly became stiffer after around 4 months.

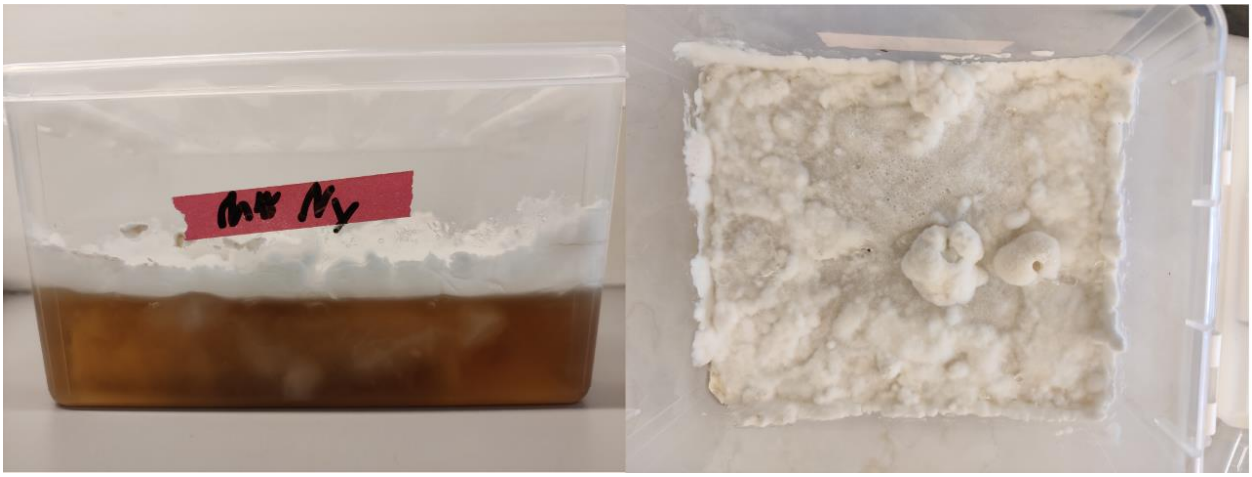

Figure 33.Picture of F.p.-5 grown in MEB on nylon tulle seen from the side (left) and seen from the top (right).

The mycelium of F.p.-5 was thicker and produced an inflexible uneven structure on the surface of the MEB, while the liquid mostly contained large amounts of loose mycelium which fell apart once moved.

The mycelium on the surface was removed from the box, partly breaking apart by even light touch. The mycelium was then placed in the wooden press and treated with glycerol. Unlike G.l.-1, F.p.-5 broke a part in the press, creating multiple smaller fragments. The glycerol treatment also seemed to slightly shrink the pieces, seen in *Figure 34.*. It had a softer and smoother texture that G.l.-1 and was not attached to the nylon.

Figure 34. F.p.5 grown in MEB on nylon tulle after it had been pressed and treated with glycerol.

Material production approach: Liquid 4- Flipping, folding, and covering.

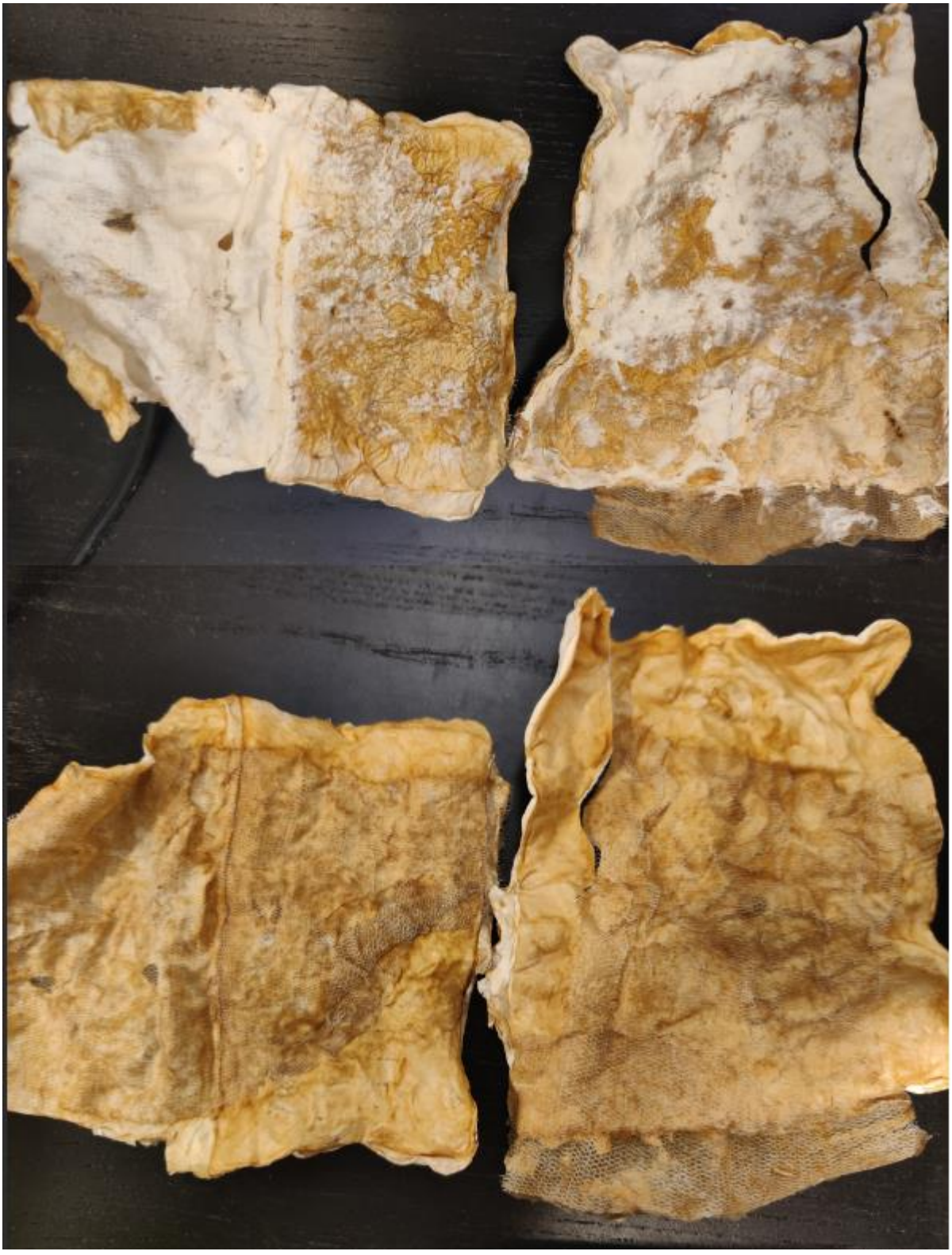

Figure 35. The left piece of mycelium sheet is a piece of G.l.-1 mycelium in which half of the mycelium had been folded onto itself creating a double layered mycelium (the right part of the mycelium) halfway through the incubation period. The right mycelium

sheet is a G.l.-1 mycelium piece which was covered by a new layer of MEB halfway through the incubation period. The top picture is from the surface facing the air, while the bottom picture is of the surface facing the MEB.

The folding of the mycelium and the covering of the mycelium with MEB lead to thicker layers of G.l.-1 mycelium being formed. In *Figure 35* the left mycelium piece was that one which had been folded onto itself. The two parts fused together during incubation, leading to the piece to be about twice as thick, reaching about 1 mm thickness. The area in which the mycelium had been removed also grew a new thin sheet of mycelium. The double layered surface is rougher, but also significantly stronger than the original single layered mycelium sheets produced in Material production approach: Liquid 3 -Boxes. This came at a slight cost in flexibility, but the double layered area could still be easily folded onto itself without causing any damage to the material.

The mycelium covered by a new layer of MEB, seen in *Figure 35* to the right, was thicker than the folded mycelium, with a less distinct layering of the mycelium, while also being more uneven, going from around 1mm thickness in the thinnest parts, while reaching around 2mm in the thickest portions of the sheet. It had a smoother surface than the folded mycelium, with a leathery texture. The flexibility was like that of the mycelium produced by the folding.

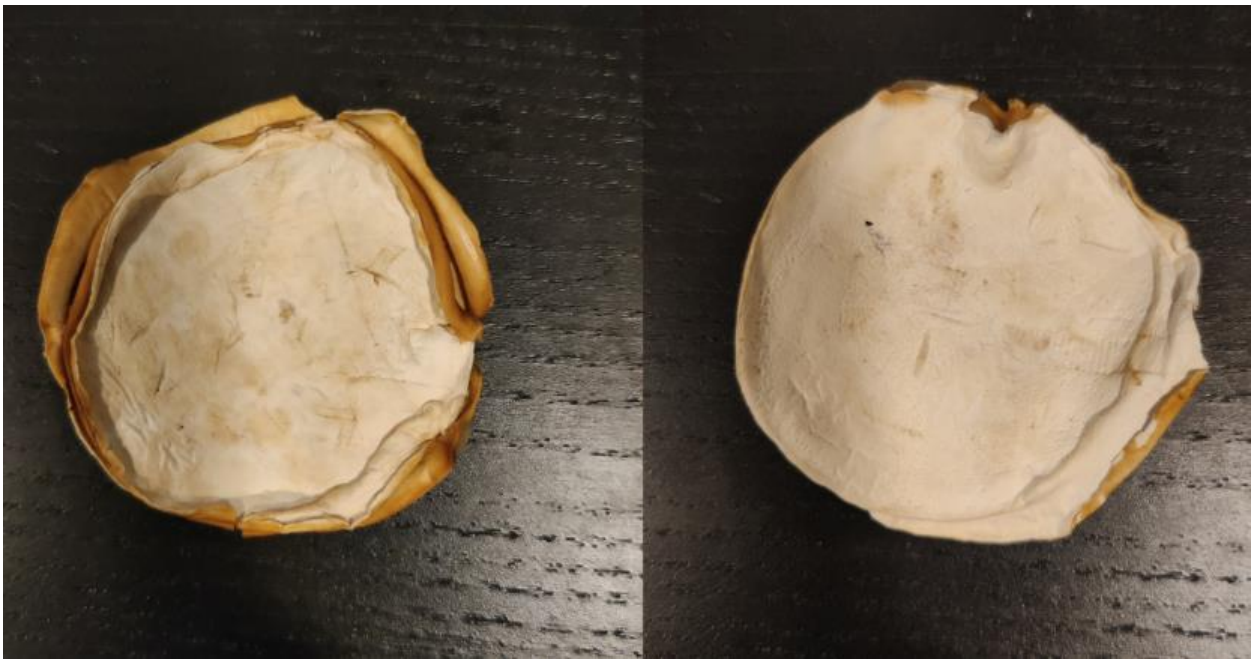

Figure 36. Picture of G.l.-1 mycelium which sheet was flipped a total of three times. The picture to the left is of the bottom of the mycelium, while the right picture is of the top of the mycelium.

The flipped mycelium pieces produced two surfaces with soft leathery white texture, with distinct folds on the edges probably caused by the shape of the Erlenmeyer flask. It seems as if the third flipping of the fungi did not produce a new sheet, which is surprising, since a new sheet of mycelium could form when the mycelium was covered by MEB. The significant difference is the access to air the new mycelium sheet had, which in this case may be restricted. The mycelium became was more resistant to mechanical stress than the mycelium sheets produced by Material production approach: Liquid 3 -Boxes, but not as much as the sheets produced by folding the mycelium pieces and by covering the mycelium pieces with MEB.

Material production approach: Liquid 5- Bioreactor

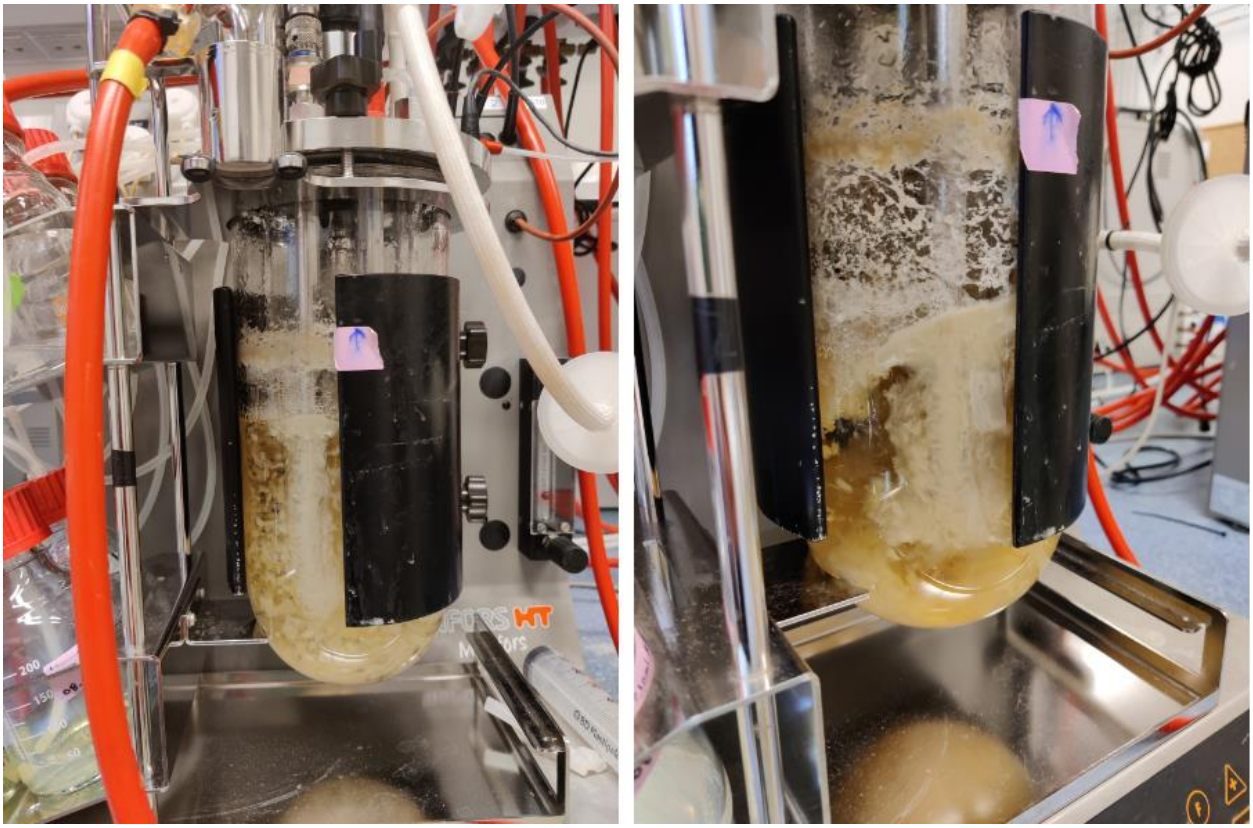

Figure 37. Picture of bioreactor after 1 week of running (left) and at the end of the two-week run (right).

The mycelium in the bioreactor took the shape of small 1x1 mm pieces which accumulated on any surface in the bioreactor which allowed attachment, and in a larger volume at the bottom. Around 200 ml culture media was lost each week, making it difficult to see if the higher concentration was mainly a consequence of growth, or if it was a symptom of the evaporation.

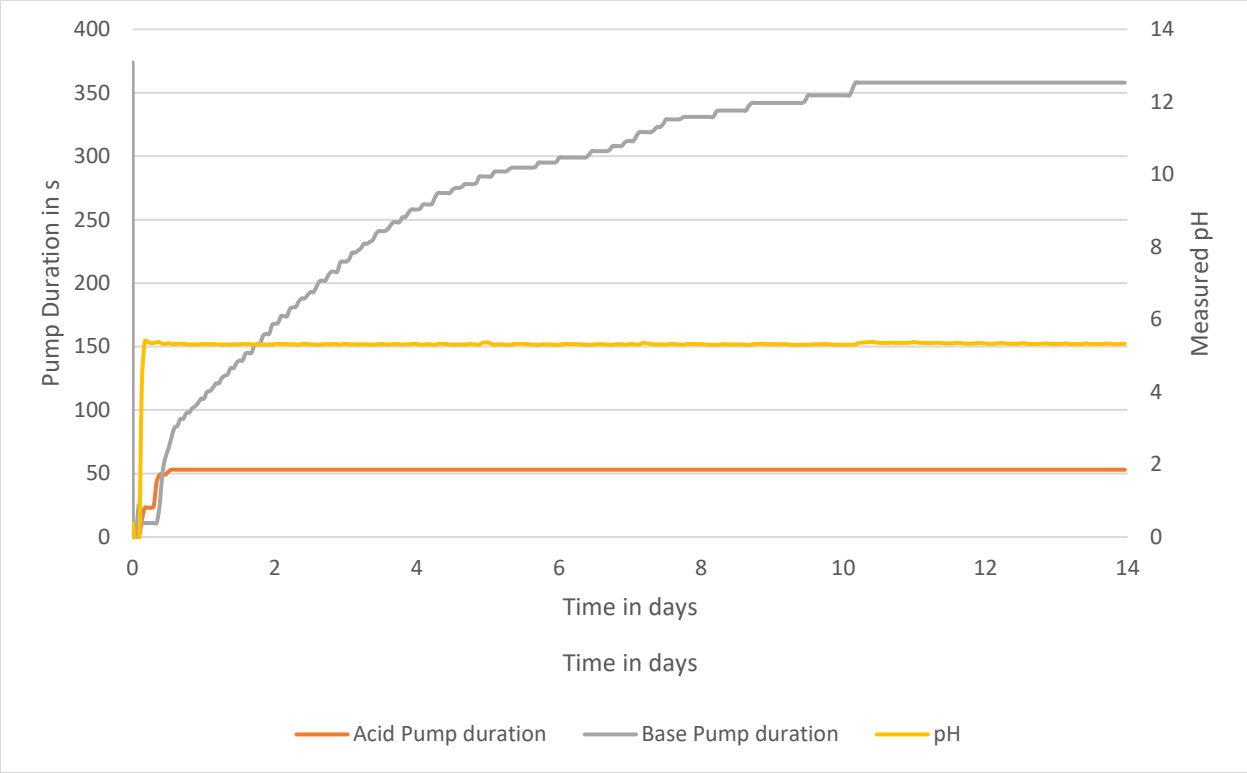

Figure 38. The change in pH measured by the bioreactor and the duration the pumps involved in the transfer of 5M NaOH and 5M H_2SO_4 were active.

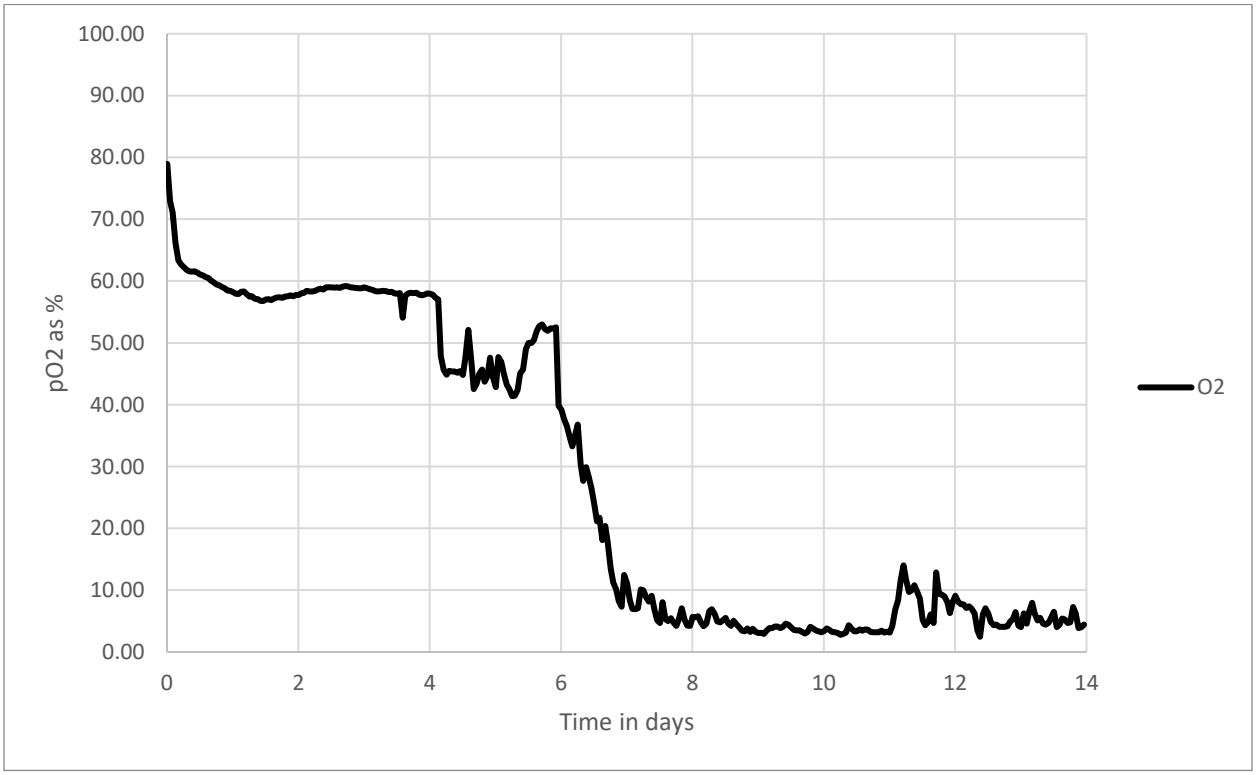

Figure 39.The partial pressure of oxygen as a % measured in the bioreactor.

There were initially problems with the pH meter of the bioreactor, which lead to continuous input of H_2SO_4, resulting in an extremely low pH as seen in *Figure 38.*. This was initially countered by the manual addition of 5 M NaOH and the manual pH measurement. The pH meter was fixed, but it was not calibrated correctly, meaning the measured pH value of 5.3 represented a pH of around 4 to 4.1. In the first few hours after the pH meter was fixed, some acid was added to maintain the pH. After 7 hours though, NaOH was added instead until 10 days had passed from the inoculation.

The partial pressure of oxygen continuously decreased as seen in *Figure 39.*. At day 5, the air flow was increased to Q_g= 1.5 air/L in response, and then to Q_g= 2 air/L at day 11. Still, the pO_2 fell to around 3-8% from day 8.

Figure 40. Fungal biomass extracted from the bioreactor.

After two weeks, the fungal biomass was extracted from the bioreactor. The biomass was composed of loose yellow to white particles with a sludgy feel. They were pressed and heat treated, which resulted in a thin brittle film covering portions of the plastic sheet it was pressed on, too thin and too inconsistent to be further treated.

Material production approach: Solid state cultivation

The rye in the mycobags were partly covered by dense white mycelium before the mycelium covered rye was mixed with sawdust. Both fungi grew poorly in the oak sawdust, and well in the spruce.

Figure 41 Picture of box containing oak sawdust inoculated with G.l.-1 mycelium covered rye.

The oak sawdust inoculated with G.l.-1 seen in *Figure 41* contained some whiteish regions which indicated higher degree of mycelium coverage of the sawdust. Overall, the sawdust had accumulated into clumps loosely connected with mycelium which were easy to break apart by hand. It did not show any promise as a material.

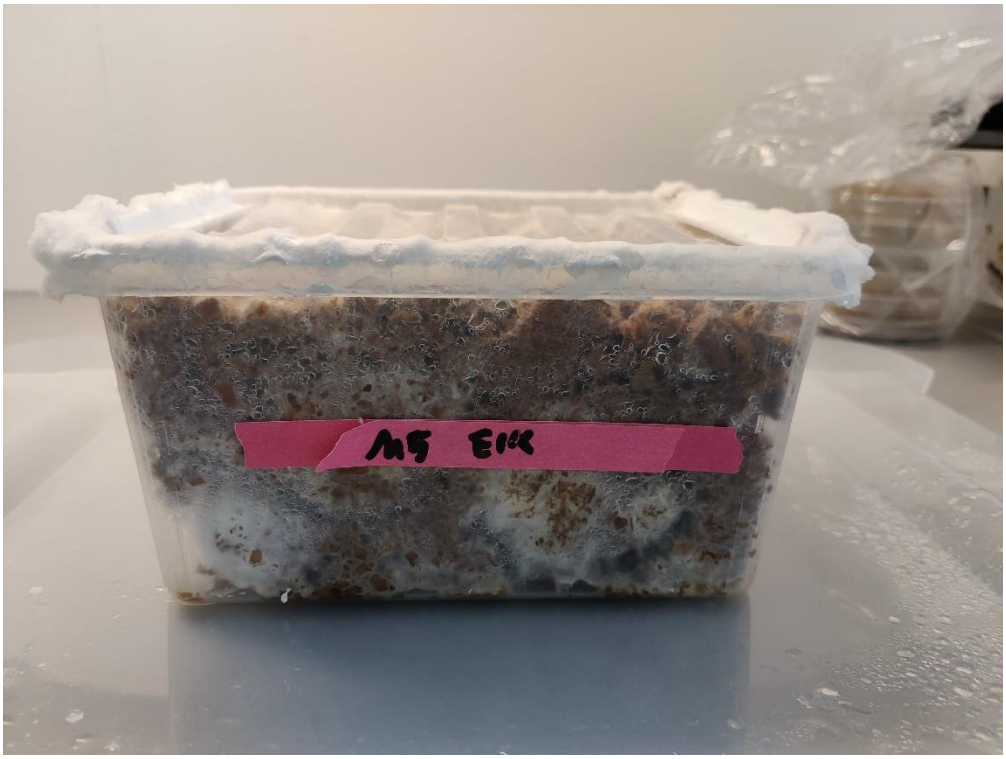

Figure 42. Picture of box containing oak sawdust inoculated with F.p.-5 mycelium covered rye.

The oak inoculated with F.p.-5 was very similar to the oak inoculated with G.l.-1, in that the sawdust had accumulated into clumps loosely held together by mycelium. The most significant difference was that mycelium had climbed the wall of the box, growing out of it through the gaps under the lid. The mycelium was also fluffier. This was also deemed useless as material.

Figure 43 Picture of box containing spruce sawdust inoculated with G.l.-1 mycelium covered rye. The left picture is taken from the side, while the right is taken from the top.

The inoculated spruce was far more promising. In *Figure 43*, we see pictures of the G.l.-1 inoculated spruce sawdust after it was heat treated. The sawdust and been connected by the mycelium, forming one 13.5 x 15.5 x 6 cm solid piece. It weighed 201.7 g, resulting in a density of around 161 g/m^3. The

surface of the mycelium was uneven, limiting the accuracy of this measurement. The compound was more resistant to mechanical stress than the mycelium infested oak but could still be broken apart by hand. The mycelium was at its densest at the surface of the sawdust, providing a thin white film which could be ripped off in small pieces.

Figure 44 Picture of box containing spruce sawdust inoculated with F.p.-5 mycelium covered rye. The left picture is taken from the side, while the right is taken from the top.

The spruce infested with F.p.-5 mycelium, pictured in *Figure 44*. was like the G.l.-1 infested spruce in that the mycelium had bound all the sawdust together, but different in that the mycelium in the F.p.-5 infested spruce covered more of the surface. The areas most covered by mycelium were soft with a slightly fluffy texture, and mostly white, with a few exceptions where the mycelium had taken on a brownish colour and a more brittle texture. Unfortunately, the density of the material was not calculated due to poor packing of the sawdust when the experience was started, which lead to a highly uneven surface. The whole structure weighed 214.7 g, indicating a similar density to the G.l.-1 infested spruce sawdust. The F.p.-5 structure could only be broken apart on weak spots on the edges. Splitting it in two by hand was not possible, meaning the mycelium provided significant structural support.

Discussion

The tendency of two fungal isolates to form sheets of mycelium when cultivated in liquid medium was here utilized to produce thicker leathery mycelium sheets through the layering of the mycelium. The two species produced mycelium with significantly different properties, indicating the value in exploring multiple species in the production of fungal leather alternatives. The G.l.-1 isolate, which was recognized to belonging to the *Ganoderma sessile* clade was the most promising isolate used in this thesis, given the highly flexible properties and significant strength of the material. The need for some physical and chemical treatment in the production of mycelium-based materials was also shown, with the press producing a more even surface, and the use of glycerol as a plasticizer, improving the flexibility of the mycelium sheets. Experiments further exploring the production of mycelium-based materials produced by growing the fungi in liquid medium should more deeply explore the varied properties of different species, and further explore chemical treatments, such as crosslinkers which may increase the strength of the materials by linking the hypha together.

Cultivation of the isolates

The experiments started with an unexpected challenge in that three of the five fungi did not grow on the plates. The cause was not discovered since the conditions on the malt extract agar plates should have been adequate for growth. Cultivation in other media, such as the potato dextrose agar, which often allows for growth of fungi, did not help either. This could indicate that the mycelium in these three fungi may already have been dead, but there is also the option that mistakes were made in the medium production, or that other conditions required for growth were not adequately taken care of. This led the experiments to be limited to only two species of fungi, or even one in some cases.

Identification of the isolates

Since only mycelium, not complete fruiting bodies was retrieved from Mycela, identification of the fungi was entirely dependent on molecular analysis. This faced some challenges, as the gDNA was difficult to isolate. The guess was that the first isolation protocol was too gentle, so harsher protocols were attempted instead. The smallest change was the smaller beads, larger tubes, and the use of the ATL lysis buffer, which contains sodium dodecyl sulphate. The result was very promising, producing large bands in the gel, without any sign of excessive shearing of the DNA. The heat shock and the dry freeze protocols also worked well, possibly by the damaging of the cell wall by the formation of ice crystals, though it should be noted that this protocol also included the same changes as the ATL protocol, so the addition of sodium dodecyl sulphate may have been the main factor. The conclusion of this section is that mycelium can be highly resistant to degradation, so harsher DNA isolation protocols must be used, but this does not prove a problem, as high quality gDNA is still retrieved.

The DNA amplification was also not without challenges, but most, except for the amplification of *tef1α* from the F.p.-5 sample, were achieved. The problem was that all the amplification protocols led to the production of two different bands. The cause was not specifically explored, if needed it could be explored by the separation and isolation of the two bands using gel electrophoresis, since the fragments had distinctly different sizes.

Fortunately, the use of only two amplicons, the ITS and the *rpb2* amplicons, were still enough to build a satisfying phylogram using the RAxML-NG algorithm, which placed the F.p.-5 within the *Fomitopsis pinicola* clade. Given that only two genes were used to build the phylogram, the confidence is not as high as desired. Tt is recommended to utilize more genes for a higher resolution if there was a demand for the identification of the strain.

The G.l.-1 isolate had a different surprise waiting. It was retrieved as a commercial *Ganoderma lucidum* strain. The amplification of ITS, *rpb2*, and *tef1α* went well, which allowed the interference of a phylogram using both maximum likelihood and Bayesian interference. This interference placed the G.l.-1 isolate into a *Ganoderma sessile* clade with a bootstrap value of 99 and a posterior probability of 100%. *G. sessile* is a fungus like *G. lucidum* in many ways, producing a reddish lacquered bracket with a white but brown bruising underside, and may in some cases produce a stem. The most significant difference between the two fungi from a common approach is where the fungi are native to, as *G. lucidum* is thought to be native to Eurasia, while *G. sessile* is native to north America, though even this distinction is weakened since *G. lucidum* has been introduced into north America[66].

Identification of fungi tends to be difficult, given the high diversity and similar structures fungi often possess. This can quickly lead to the misidentification when molecular techniques are not used, or when

the techniques are lacking or misused. Given the rate the fungal taxonomy is changing[67], any identification should be accompanied with adequate amounts of distrust. The misidentification of fungi is overall most harmful when the fungus is eaten, given the ability of many fungi to produce toxins, care should especially be taken in industry and research. In industrial applications, the use of misidentified fungi, especially molds, may result in the widespread distribution of products contaminated with toxins. Within research, the misidentification of fungi may harm the ability of other researchers to repeat and further develop on the experiments. The researchers may also be exposed to harmful compounds without their knowledge. In the production of mycelium-based materials, the exposure to toxic compounds is likely less problematic, given that the product is not digested, but care should still be taken.

Liquid incubation

The investigation of the potential material started with cultivating the mycelium in petri dishes. The problems were immediately obvious, with the liquid disappearing quickly, most likely due to evaporation. This was necessarily easy to solve by simply increasing the volume of the growth medium. It also indicated that the fungi may benefit from having a structure to form biofilm on, such as the rice paper containing plates had more developed mycelium, though this is not necessary and can be resolved by increasing the incubation times. Another aspect was the need for extra compounds in the fungi to prevent the mycelium from simply turning into a brittle sheet once dried. Luckily, this is not a problem unique to fungi, but is a well-known part of producing materials with desired flexible properties. The compounds used to solve this problem are called plasticizers. These compounds are low-molecular weight molecules non-volatile compounds that may integrate between the polymers, increasing the space, allowing them to move more freely without damaging the material [68]. This was well demonstrated by the addition of glycerol to the mycelium, which completely changed the mycelium from brittle sheets to material with leather-like properties. They may also have the additional property of increasing the hydrophobic properties of the material, an interesting property which would be highly desired in an alternative leather product. Finally, increasing the thickness and strength of the material is a significant challenge.

With the second liquid incubation experience, a few properties became very clear. One of the goals with the experiment was to allow the fungi to grow through the t-shirt, hopefully combining the properties of the cotton with some of the properties with the mycelium. This did not happen, initially thought to be only because of the pores in the t-shirt being too small for the mycelium to effectively pass through, though it would also become clear that this was not the only factor in the next liquid incubation experiment. Another thing that was clearly demonstrated is that the properties of mycelium significantly differ between taxa. The *G. lucidum* mycelium was leathery and was more resistant to mechanical strain. The *F. pinicola* mycelium had a slightly soft feel and was also very vulnerable to being ripped apart. There may be significant value in investigating many fungi from different taxa as some species may have interesting properties. Another interesting note was that it seemed like the *G. lucidum* had some ability to degrade the t-shirt and integrate the black dye into the mycelium. This may be of interest in material production, as the dye can be integrated far more deeply into the structure than simply painting the end-product mycelium sheets. It also indicates that it may be practical to integrate other desired compounds into the mycelium already in the incubation process if it doesn't harm the growth of the fungi. It should also be noted that the *F. pinicola* mycelium did not integrate any dye into its mycelium.

This may be due to the mycelium not degrading the t-shirt cotton at all, or that the integration of compounds can differ.

An aspect that became clear with the second liquid incubation experience was that an easier approach to incubation would be beneficial. The utilization of boxes with lids for the incubation and the use of nylon tulle were good initial solutions to this. The boxes had bigger surfaces than the engineered incubation chambers, while the ability of nylon to float made the possible need to adjust the height of the mycelium support unnecessary. The nylon tulle also had large holes, which would allow the fungi to freely grow through them. A hope in the beginning was that the mycelium would integrate the nylon into its structure, but this only happened to a very limited degree. The nylon tulle would consistently function as a base of the mycelium sheet and could be ripped off with little effort. The benefit of this is that the nylon could assist in the growth of the fungi by allowing the mycelium to have a structure to attach to while growing, and then be removed and recycled after the mycelium sheet had grown. The third liquid incubation approach once again indicated the important differences in the taxa. The *G. lucidum* mycelium sheets were far more even and more structurally sound than the *F. pinicola* sheets, which fell apart when the liquid was drained from the boxes. Another observation was that the glycerol treatment didn't function as in previous experiments, which may be due to the thicker mycelium sheets.

The layering and folding of the mycelium sheets were probably one of the largest developments in this paper. These approaches could easily increase the thickness of the material, thereby also granting more strength. It is still unfortunately possible to rip the material by hand, given enough force used. A repeated folding approach or the addition of media in multiple layers may further improve the thickness and strength of the material, but this comes at a cost of time, and may affect the flexibility of the material more than it does the strength. In its current state, the layered mycelium may be used to produce some more inflexible products such as bags, but these products would likely be more of a proof of concept than a product that could compete with either synthetic or real leather.

The result of flipping the mycelium sheets was also interesting, less in that it improved the strength of the material, but more so that each side of the product was identical, a property which may be desirable in some cases.

To further strengthen the mycelium sheets, alternative approaches may be worth examining. In this case, I suggest exploring the introduction of some fibrous material between the folded layers to act as a scaffold for the mycelium. This product could be a porous nylon sheet, or possibly a cellulose sheet if desired to keep the product entirely oil free. The chemical treatment of the sheets should also be explored. Some papers have suggested the crosslinking of the hyphae, for example using genipin, a crosslinker found in *Genipa america* capable of crosslinking multiple compounds, including proteins and chitosan [69]. Crosslinking seems to have mainly been explored in the crosslinking of loose biomass, where the results have been, not in the crosslinking of already formed mycelium sheets, which may provide radically different results [70]. Tanning processes have also been used to improve the qualities of the material, where the tannins function by binding proteins, increasing the strength of the material. It should be noted here, that the use of tanning method should be carefully considered, as not to simply imitate the problematic processes used in conventional leather production using chrome tanning [71].

Further research should also utilize tools to measure the tensile strength and elongation break percentage to better compare the results of this research to previous methods.

An alternative liquid technique was approached using the bioreactor. The goal was to produce larger amounts of mycelium, but some challenges were encountered. The experiment started with the failure of the pH-meter, which led to a large input of H_2SO_4, which had to be balanced out with NaOH, adding salts to the bioreactor. This did not stop the mycelium from growing though, probably most shown by the oxygen consumption in the bioreactor. The G.l.-1 also seemed to continuously produce an acid, which necessitated the input of NaOH to maintain the pH.

As common with filamentous fungi, the fungi accumulated on every surface it could attach to, but due to the continuous stirring, no significant film formed, restricting the mycelium to tiny pieces. Therefore, any material production using this biomass would probably require significant post-cultivation treatment.

Comparison to other fungal leathers

The products of the liquid cultivation experiments seem to resemble leather most closely. This is not new, as the filamentous fungi have been used to produce leather alternatives already. Unfortunately, many of the details of the methodology in other technologies are hidden behind patents.

One example is VTT Technical Research Center in Finland, which seem to use bioreactors to generate biomass, though the growth conditions are unknown. Their approach to the post cultivation steps seems far more advanced than explored here, using a series of washing, filtration, crosslinking steps, in addition to other potential treatments to fuse to likely loose biomass produced in their bioreactor into a leather alternative [70].

The other seemingly widely used approach is the inducing of aerial mycelium growth. A solid growth substrate is inoculated with a fungal species fit for the substrate. The inoculated medium is then stored in a controlled atmosphere, with suggestions encountered being a 3-20 % CO_2 high humidity atmosphere. This seems to discourage the fungi from developing fruiting body, while allowing the mycelium to grow out of the substrate. The mycelium can then be removed from the surface of the substrate, further treated with tannins, crosslinkers, wax, dyes, or other desired products [70, 72].

Solid state incubation

The cultivation of the fungi in the sawdust produced some interesting results. When it came to the incubation, the different growth in oak and spruce sawdust indicate that the growth medium has a large impact on the resulting material, with oak not producing anything viable in this experiment, while the spruce sawdust produced lightweight blocks of a mycelium composite material. It should also be noted that while in the liquid incubation experiments, *F. pinicola* did not perform well, it produced a far stronger material when used for composite production. This may be due to the *F. pinicola* growing denser through the sawdust than the *G. lucidum* did, but there may also be other causes not explored. Unfortunately, the solid-state incubation did not receive much more attention here, and the exact uses of the composites was not really investigated. Some previous research has suggested the use as fire retardant insulation, as noise absorbing panels, or as packing material[73].

Conclusion

By growing certain fungi in liquid media, leather-like sheets of mycelium can be produced. While initially thin, enhancing the strength and thickness of the material is possible simply by layering the mycelium, either by flipping it, folding it, or covering it with new growth media. Further improvements to the

quality of the material can likely be approached through testing of different species of fungi, and further post treatment of the mycelium sheets.

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