THESIS

DEVELOPMENT AND CHARACTERIZATIONS OF MYCELIUM-BASED COMPOSITES

Submitted by

Morgan N. Davis

Department of Design and Merchandising

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Summer 2024

Master's Committee:

Advisor: Yan Vivian Li

Kristen Morris
Jane Stewart

ABSTRACT

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The current materials economy produces linear materials without regard to their end of life. As the demand for these materials rises, the demand for a new textile economy increases: regenerative by design, minimizing resource input, and waste systems. Mycelium-based composites (MBC) is a lightweight biodegradable material, with good thermal insulation, fire resistance, and acoustic attenuation. The role of MBC lies at a crossroads between biology and design, where designers incorporate biological principles and need to understand the mechanisms of material growth. The study's specific aims include the evaluation of the chemical, mechanical, and physical properties of MBC developed in various conditions and nutrient substrates. Essential to this process is the cultivation of the mycelium, where careful considerations of fungal species, nutrient substrate, and growing parameters are critical. A component of the first critical factor emerges: verification of the fungal DNA. The nutrient substrate hemp fabric demonstrates great compatibility, with the fungal species *Pleurotus fossulatus,* as it has the largest hyphae diameter, and offers solutions to incorporate waste. In contrast to the strong hydrophilic nature inherent in many natural cellose, mycelium exhibits hydrophobic properties, a critical feature in terms of product implication. The results provide insight for the future design and optimization of mycelium-based composites for product development innovation.

ACKNOWLEDGMENTS

I would like to sincerely thank the faculty and staff in the Department of Design and Merchandising for their involvement and role in my successful completion of this M.S. degree. From my professors who taught me how to read a research article, those who encouraged me to lean into my interests (even if it is scary and new), and those who shared words of encouragement with me while we were passing in the hall. You all have made these past two years in Colorado a memorable, meaningful journey and I am thankful for you all!

To my husband who took a leap of faith moving across the country so I could complete this degree, THANK YOU. For allowing me to follow my dreams, no matter what, and supporting my mental and physical health along the way!

To my advisor, Dr. Li, thank you for taking me on a semester early for an independent study where I could dive deeper into my interests and develop the groundwork for my project. Thank you for all the hours we spent in your office brainstorming and working on how my project. Thank you for your dedication! To Dr. Morris, as my internal committee member and also my GTA/GRA advisor, you have taught me so much about the teacher and research I strive to be. I hold you in the highest regard of role models, you have truly made such a valuable impact on my life. Thank you for all you have done for me, from my first conference, to my first award, and much more! I will miss our 'work parties' very much! To my outside committee member, Dr. Stewart. Thank you for all your valuable insight and contribution to this project. I have learned so much about fungus and mycelium.

Finally, thank you to my friends and family who have supported me from the beginning of this journey! Your support is invaluable and means so much to me!

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Chapter 1 Introduction and Background

The primary goal of this research study was to develop and characterize mycelium-based composites and explore their potential application in biodesign and textile product development. The study's specific aims included the evaluation of the chemical, mechanical, and physical properties of mycelium-based composites developed in various conditions and nutrient substrates.

As the population increases so does environmental pollution, generation of waste, and depletion of natural resources (Alemu et al., 2022). Annual waste generation is expected to increase with predicted values of 2.2 billion tons in 2025 to 3.4 billion tons in 2050 (Alemu et al., 2022). The major sources of these wastes are commercial centers, construction sectors, and domestic agriculture (Alemu et al., 2022). The physical goods economy produces linear materials by extracting finite valuable resources without regard to their end of life and environmental impact (Elsacker et al., 2019). Production, transportation, and disposal of these materials consume energy, limit natural resources, and pollute air, soil, and water bodies (Alemu et al., 2022). With the need for these materials continuously increasing, the pressing issue calls for alternative ways of using existing resources focused on renewable and recyclable materials (Alemu et al., 2022).

Fungi derived, mycelium-based materials are natural polymeric composites that require minimum energy for production, due to their self-growing capability, and their characteristics can be tuned by modifying their nutrient substrate (Haneef et al., 2017). This material is lightweight, biodegradable composite, with low environmental impact with the potential to replace fossil-based and synthetic materials such as polyurethane and polystyrene (Cerimi et al., 2019 & Elsacker et al., 2019). Patent developments for mycelium materials suggest that the

bio-based material will considerably shape the future of material sciences and material applications (Cerimi et al., 2019).

A new textile economy is reimagined based on principles of Circular Economy (CE), regenerative by design, and provides benefits for business, society, and the environment (Ellen MacArthur Foundation, 2017). A Circular Economy is a regenerative system that minimizes resource input and waste systems by narrowing and closing material and energy loops (Geissdoerfer et al., 2016). This is achievable through long-lasting design, maintenance, repair, reuse, recycling, remanufacturing, and refurbishing (Geissdoerfer et al., 2016). See Figure 1.

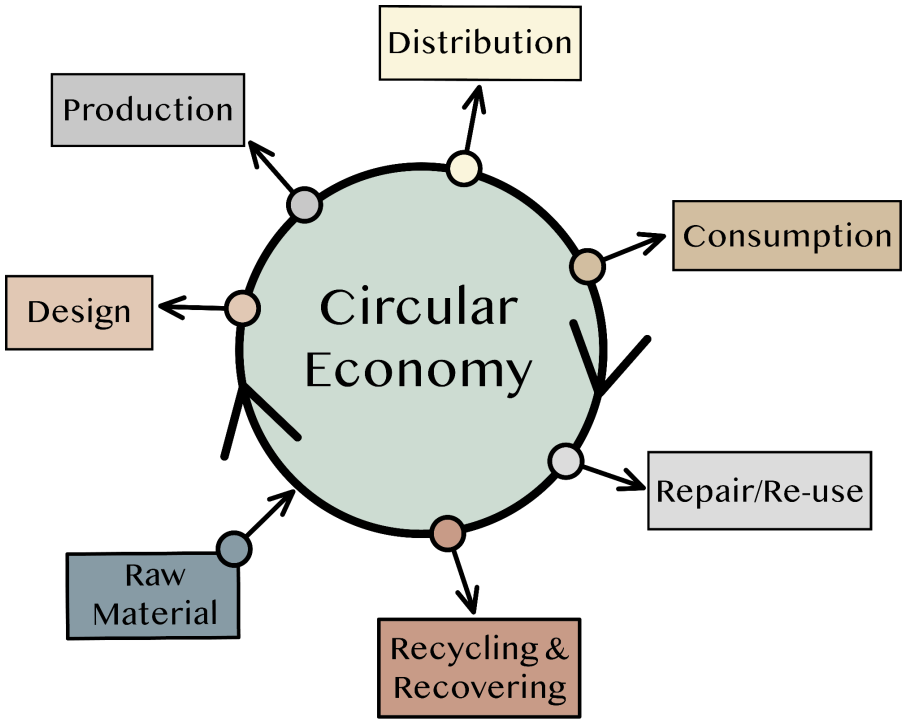

Figure 1 Model of a product's life cycle in a circular economy.

The main motivation for CE is to shift away from the traditional linear production model to a circular model where resources and wastes are better used (Geissdoerfer et al., 2016). A systematic change consists of making better use of resources and moving to renewable inputs, where production generates less waste, reduces water use, and requires fewer inputs of

nonrenewable resources (Ellen MacArthur Foundation, 2017). Nature's circular systems act as a model for the systematic change that is needed (McDonought & Braugart, 2002). Recovery is essential for CE and should be considered before production begins (Niinimaki, 2018). Mycelium-based composites have eco-friendly characteristics such as the usage of waste materials and low energy needed during production(Sydor et al., 2022). The production does not generate waste, and the products are easily recycled (Sydor et al., 2022).

Mycelium-based composites (MBC) biodegrade their substrates to form a material that can be used as an eco-friendly textile (Vašatko et al., 2022). MBCs are lightweight, foam-like material that is nontoxic, 100% biodegradable, and offer solutions for post-consumer and industry waste (Sydor et al., 2022 & Vandelook, 2021). Mycelium offers strategies for sustainable production methods, where the materials in the substrate are as a dual benefiting waste management system. The mycelium is getting the nutrients it needs for colonization and degrading post-consumer waste.

MBC performance properties are typically inferior to the materials currently in use, however, MBC materials show high acoustic attenuation, thermal insulation, fire resistance, and the absence of harmful synthetic chemical components (Cerimi et al., 2019 & Sydor et al., 2022). To strengthen the material's performance properties, a holistic understanding of mycelium materials' chemical composition, nanostructure, and relationship with water was needed to design a quality circular product. This understanding shaped the research hypothesis and objectives. The fungal species, nutrient substrate, and growing parameters are important factors to understand in developing a quality material (Sydor et al., 2020). The fungal species *Pleurotus ostreatus (P. ostreatus*) is recommended for future development by many research reports, noting its high compressive strength, wide availability, and low cost (Silverman et al., 2020; Sydor et

al., 2022; & Vašatko et al., 2022). Researchers have suggested further research with the nutrient substrates of cardboard, applewood, and natural fibers as reinforcement, as these nutrient substrates have shown high compression strength, colonization density, and minimized waste (Attias et al., 2017; Silverman et al., 2020; & Vašatko et al., 2022).

Imaging is an important for characterizing a materials mechanical strength and for understanding how the fungus interacts with the nutrient substrate. For imaging the Scanning Electron Microscope (SEM) was used. Understanding the material's relationship with water allows for a thoughtful analysis of the material's end use. To determine the material's hydrophobicity, Theta Flex was used to produce the material's corrected contact angle.

To achieve a material with high compression strength, compressing the wet material to tightly pack the sample early in the growing stages is suggested, similarly inconsistent pressure from manually filling has been shown to affect the uniformity of the sample in shape and density (Silverman et al., 2020 & Vašatko et al., 2022). To account for the inconsistent pressure and compression during growth stages, periodic compression with a 3D-printed lid was used during inoculation and colonization. Using Fourier-transform infrared spectroscopy (FTIR) analysis, Haneef et al., 2017 showed that the material's mechanical strength is closely related to the nutrient substrate the mycelium is digesting allowing for the mechanical properties to be fine-tuned. Tinius Olsen will be used to conduct tensile strength testing for dermining how the strength of the hemp fabric was affected by the mycelium colonization. These parameters were used to uncover the material's chemical, mechanical, and physical properties for strengthening the material's performance and broadening the material's application. These applications range from architecture, packaging, the automotive industry, and furniture materials (Sydor et al., 2022).

Hypothesis

Mycelium-based composite (MBC) performance properties can be increased by using hemp fabric, cardboard, and applewood chips as nutrient substrates, and compression during inoculation and colonization.

Study Objectives

1st Objective: To develop a replicable protocol for producing mycelium-based composites by identifying growing parameters during manufacturing.

2nd Objective: To utilize morphological imaging for pure and composite mycelium materials.

3rd Objective: To evaluate mycelium materials in terms of chemical, mechanical, and physical properties and explore their potential application in biodesign and textile product development.

Chapter 2 Literature Review

Synthetic fibers such as nylon, polyester, polypropylene, and acrylic, are known pollutants produced by the fashion industry (Muthu et al., 2012). These fibers are non-biodegradable, made with nonrenewable resources, and have high CO2 emissions (Muthu et al., 2012). Washing synthetic fibers causes the shed of microfibers, a fiber that is less than 5mm and is considered a microplastic (Gavigan et al., 2020). These microplastics are small enough to fit through a washing machine filter and are released into waterways (Gavigan et al., 2020). It is estimated that 5.6 megatons of synthetic microfibers were emitted from washing apparel between 1950 to 2016 (Gavigan et al., 2020). Humans inhale airborne microfibers and ingest them in commonly found foods such as water, alcohol, seafood, sugar, and honey (Gavigan et al., 2020). Natural fibers are thought to be better for the environment, but their impact can not be ignored. Cotton requires a large amount of water and chemicals to be produced, in addition to significantly damaging the land (Wood, 2019). The Aral Sea in Central Asia provides an example of the outcome of overfarming (Wood, 2019). The region was once a rich and fertile land but has turned into desert-like conditions due to the overfarming of cotton (Wood, 2019).

Outside the textile and apparel materials industry, other industries are producing materials with significant environmental impact, such as construction and packaging materials (Alemu et al., 2022). Plastic pollution is a growing environmental issue (Gavigan et al., 2020). Polystyrene, also known as styrofoam, is a thermoplastic substance that is not biodegradable or compostable (Abhijith et al., 2018). Standard packaging materials are mainly petroleum products, which have various disadvantages such as high energy consumption during production, difficulty regarding degradation, and environmental pollution (Cerimi et al., 2019). Due to the need for developing new green sustainable materials, biological systems are being used in

material science (Haneff et al, 2017). Environmental degradation issues of synthetic plastics in combination with fossil depletion are the main reasons for research in materials obtained from renewable resources (Haneef et al, 2017).

2.1 Fungus in Nature

Mycelium, a component of fungal growth, plays an essential role in our ecosystem (Stamets, 2005). Fungi are the grand recyclers of our planet, and they dissemble large organic molecules into smaller, simpler forms, which in return, provide nourishment to other organisms while healing and repairing our shared environment (Stamets, 2005). Mycorestoration is a term coined by Paul Stamets (2005) in his book *Mycelium Running.* Mycorestoration describes the act of using fungi to improve the health of the environment (Stamets, 2005). There are a variety of successful uses demonstrated by Stamets (2005) such as mycofiltation: to reduce and catch upstream contaminants, mycoforestry: to enhance forest health, mycoremediation: to neutralize toxins, and mycopesticides: to influence and control pests. The use of mycelium to heal the environment is not a new idea but is relatively new as a circular material.

2.2 Fungus and Biodesign

Alternative material options are explored for design and manufacturing, with an emphasis on renewable materials so we do not deplete our natural resources faster than we can renew them (Collet, 2021). Bio-based materials are defined as “a material of which one or more of its components are sustainably grown and are fully renewable” (Lelivet et al., 2015). New design practices are developing to incorporate biological principles and tools designed by how nature operates (Collet, 2021). A textile made from mycelium is considered a bio-based material.

Bio-design is an emerging design practice because of its sustainable green principles, offering solutions to global waste (Collet 2017, 2021, & Wood 2019). The role of mycelium as a

material lies at a crossroads between design and biology. This intersection is prompted by a design paradigm shift, where designers are seeking living entities such as mycelium rather than traditional inanimate matter such as plastics and metals (Collet, 2021). This opens new possibilities for fabricating intelligent materials that focus on new sustainable processes (Collet, 2021). Mycelium materials have sustainable qualities that aid bio-design by offering alternatives to plastics and solutions to waste (Collet, 2017). Working as a co-designer with mycelium offers solutions to repair and heal the environment.

2.3 Mycelium Materials

In order to understand the composition of mycelium as a material, it is important to understand how the mycelium grows intertwining itself, resembling a non-woven material (Lelivet et al., 2015). Hyphae are small branch-like strands that form a dense mycelium network (Lelivet et al., 2015). The network grows and binds together to create a mycelial mat (Stamets, 2005). The mycelium absorbs its substrate and transforms it into a composite material (Collet, 2017). The feeding fungus acts as a biological glue for the material by colonizing and binding the loose waste together (Vandelook et al., 2021). MBC's use as a material is a growing body of research (Sydor et al., 2022; Vašatko et al., 2022; & Wood, 2019). MBCs have been explored in the packing industry and have proven to be a sustainable replacement for polystyrene (Abhijith et al., 2018; Afrin & Yusuf, 2013; & Wood, 2019). The production of MBC produces ten times less carbon dioxide (CO2) and about eight times less energy than the production of polystyrene foam (Afrin & Yusuf, 2013). In addition, MBCs are used for other applications such as lamp shades, flower pots, surfboards, insulation material, and shoe soles (Abhijith et al., 2018 & Silverman et al., 2020). MBCs offer value-added through low cost, low emissions, and recyclability (Alemu et al., 2022).

Mycelium is a natural polymeric composite fibrous material mainly composed of natural polymers such as chitin, cellulose, proteins, etc. (Haneef et al., 2017).The integration of composite materials with mycelial growth enhances the ability to find tune and control for the structural and mechanical properties. This is achievable through exploiting different nutrient substrates for hyphae growth, the mechanical properties of mycelium materials are closely related to their nutrient substrates (Haneef et al., 2017). Haneef et al., 2017, showed that the differences in the mycelium nutrient substrate reflected alterations in the morphology and mechanical properties. Using FTIR analysis, the researchers found that the materials grown on cellulose contained more chitin and had a higher Young's modulus and lower elongation, indicating that mycelium materials get stiffer when their feeding substrate is harder to digest (Haneef et al., 2017). All the mycelium materials were hydrophobic with water contact angles higher than 120° (Haneef et al., 2017). In addition to the nutrient substrate, growth parameter variation can result in changes in the material mechanical properties (Elsacker et al., 2019). Elascker et al., 2019 suggest the main factors affecting the production of mycelium composites, and consequently their mechanical behaviors are: fungal species, nutrient substrate, and process variables during manufacturing: protocol, sterilization, inoculation, packing, incubation, growing period, and drying method.

2.4 Critical Factors for Developing MBC

As a living organism produces mycelium, it is essential to understand the conditions to grow and the process of transforming it into a composite material. Researchers can agree that there are three categories of variables that affect the success of the material. Recent basic research has shown that 1. Fungal species, 2. Nutrient substrate, and 3. The development of growing parameters (or process variables during manufacturing) are the top critical factor

affecting the composition and use of MBC, see Figure 2 (Elsacker et al., 2019 & Sydor et al., 2022). The following three sections will investigate each critical factor to provide a foundation for the methodology of the MBC materials experiment.

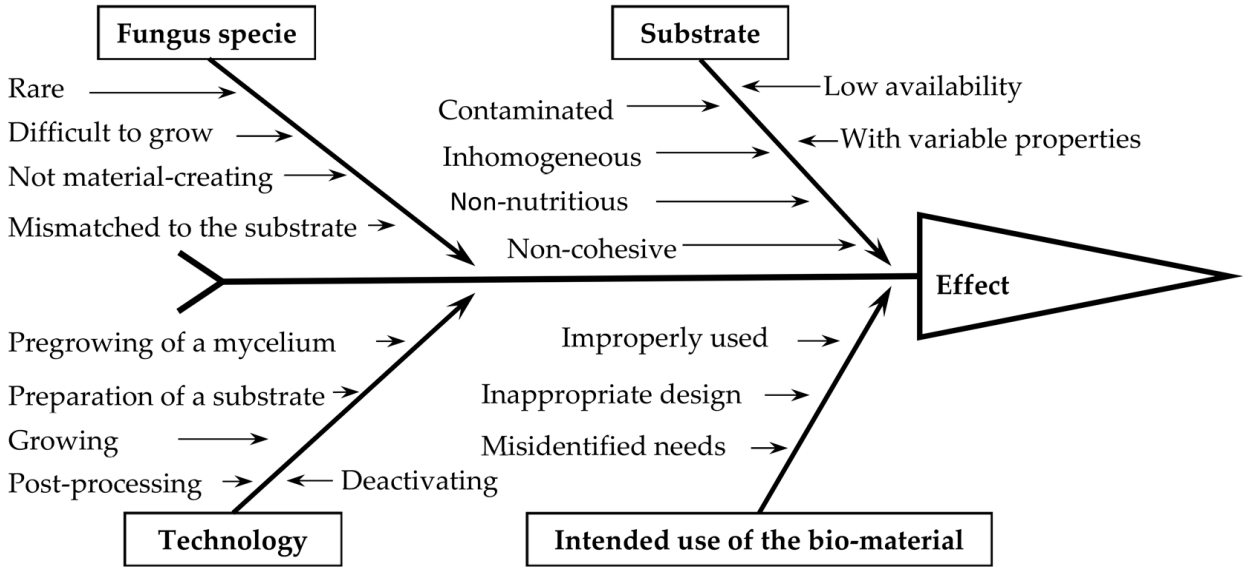

Figure 2 Factors affecting the manufacture and use of mycelium-based composites (Sydor et al., 2022).

2.4.1 Fungal Species

The Phylum Basidiomycota is typically selected for biomaterial production, specifically because of the mycelium's natural adhesive property and its ability to degrade lignocellulose (Alemu et al., 2022). During the generation of colonization, the hyphae fuse with each other, creating a network system where nutrients are transported and the mycelium can grow fast, strong, and dense (Alemu et al., 2022). The most commonly used species for mycelium materials that belong to the Phylum Basidiomycota are *Pleurotus ostreatus (P. ostreatus*) and *Ganoderma lucidum (G. Lucidum)* (Alemu et al., 2022). Both are white rot fungi and were found by a basic research study to be the most frequently used fungal species in MBC research, *Pleurotus ostreatus* (oyster mushroom) and *Ganoderma lucidum* (reishi mushroom) respectively (Sydor et al., 2022). White rot fungi act differently than other wood-degrading fungus because they have

the capability to excrete a variety of enzymes, some of which are able to degrade plant components difficult to hydrolyze, like lignin in the plant cell wall (Haneef et al., 2017 & Sydor et al., 2022). Lignin acts as a chemical adhesive between fibers, adding strength and rigidity to cell walls (Sydor et al., 2022).

P. ostreatus is a primary decomposer (Stamets, 2005). Primary decomposers are fast-growing, their mycelium quickly branches and readily decomposes its substrate (Stamets, 2005). In addition to its frequent use in MBC research, *P. ostreatus* is easily accessible and nontoxic (Helberg et al., 2019; Lelivet et al., 2015; Silverman et al., 2020; & Vašatko et al., 2022). Choosing an edible mushroom species ensures the MBC is nontoxic and can be safely biodegraded (Silverman et al., 2020). *P. ostreatus* is recommended for future development by many research reports, noting its high compressive strength, wide availability, and low cost (Silverman et al., 2020; Sydor et al., 2022; & Vašatko et al., 2022).

2.4.2 Nutrient Substrate

Typically, the substrate material is from three sources: agricultural by-products, industrial waste, or post-consumer waste (Sydor et al., 2022). These substrates can range from annual plants, to softwood, and hardwoods (Sydor et al., 2022). Pinewood sawdust was found to be the most commonly used substrate material, followed by fibrous plants with high cellulose content such as hemp, cotton, and wheat straw (Sydor et al., 2022). Mycelium substrates can also incorporate waste from the food industry, Silverman et al. (2020) demonstrated this by integrating natural textiles and chicken feather waste into their substrate. Using SEM, they demonstrated how mycelium will bind to the waste while also feeding itself with the nutrients provided by the waste (Silverman et al., 2020). This offers promise for minimizing consumer waste (Wood, 2019). The hyphae absorb the nutrients provided by the substrate in order to grow

and form a dense network (Silverman et al., 2020). To increase the strength of the MBC material, it is recommended to include long-fibrous strands in the substrate, such as wood chips or straw (Sydor et al., 2022). While incorporating different materials in the substrate offers an opportunity to enhance the mechanical features, it also offers opportunities to enhance the aesthetic features of the MBC. Sydor et al. (2022) demonstrated this by incorporating eggshells, which resemble a confetti pattern, or 3D wood cubes, to give it an interesting hand and geometric look.

For the mother spawns (the first generation of mycelium produced with the liquid culture) nutrient substrate, rye berries are the choice of most spawn makers because rye is low-cost and allows for the ability to separate into individual kernels (Stamets & Chilton, 1983, 2000). Throughout the entire production process sterilization and limiting exposure to contaminants is highly important. In a single gram of rye, it is an estimated cell count of 50,000-100,000 bacteria, more than 200,000 actinomyces, 12,000 fungi, and yeasts (Stamets & Chilton, 1983, 2000). To sterilize one gram of grain would kill more than 300,000 contaminants (Stamets & Chilton, 1983, 2000). Specifically for rye grain, sterilization needs to be long enough for the steam to penetrate small air pockets and structural cavities in the grain (Stamets & Chilton, 1983, 2000).

To increase the performance properties of the material, an understanding and implementation of the nutrient substrates need to be compatible with the chosen fungal species. Attias et al., 2017, compared substrates from eucalyptus, vine-cabernet sauvigon, apple, pine, and oak wood chips as substrate material, and *P.ostreatus* grown on apple or vine wood had the best results for quantitative change in organic matter content during mushroom growth with the qualitative parameters of mycelium density and thickness. Vašatko et al., 2022, recommend cardboard in the substrate due to its excellent compression strength and colonization density,

while also biodegrading a substrate that is readily available, they note that further research using cardboard in the substrate is recommended. Integrating cellulose textile waste offers a solution to minimize waste from the textile and apparel industry and reinforcement to the composite (Silverman et al., 2020). Production scraps, remnants, or fabric from apparel at the end of its life can be integrated as reinforcements in the composite. The nutrient substrates of hemp fabric, applewood chips, and cardboard will be implemented.

2.4.3 Development of Growing Parameters

Manufacturing parameters for MBC include careful consideration of the mycelium growing time and provided conditions. These include inoculation temperature, availability of light, pH and moisture content of the substrate, and material drying method (Sydor et al., 2022). To avoid contamination, the substrate and any materials used in inoculation need to be properly sterilized. Growing time depends on the dimensions of the mold and type of fungus, ranging from 5 to 42 days, with the optimal temperature ranging from 70°F to 86°F and humidity ranging from 80% to 100% (Sydor et al., 2022). To form dense mycelium and prevent fruiting bodies mycelium should be grown in the dark (Lelivet et al. 2015). Mycelium requires oxygen and produces CO2 during growth, so it is necessary to provide fresh airflow (Lelivet et al. 2015). At the end of its growth, the mycelium is dried which prevents any future growth and turns the living mycelium into a material (Lelivet et al. 2015).

Production technology explores the ability to shape mycelium by growing it in a plastic mold and incorporating different technologies, such as 3D printing. The substrates of MBC are flexible and able to be grown to a shape in a plastic mold, therefore mycelium is a shapeable material (Weiler et al., 2019). Recent research has demonstrated the compatibility of mycelium growth in a mold for product design and development (Abhijith et al., 2018; Silverman et al.,

2020; Tang et al., 2018; & Weiler et al., 2019). Silverman et al. (2020) investigated MBC for sustainable production of footwear products, resulting in an MBC as a shoe sole. The MBC's properties were similar to a stiff, tough cork and showed high compressive strength, demonstrating applicability for shoe soles (Silverman et al., 2020). Similarly, Tang et al. (2018) designed a sandal shoe, the MBC shoe sole was incorporated to show the utilization of mycelium-based composites for shoe sole applications**.**

New technologies such as 3D printing are being investigated to understand the role they can play in the mold-making process (Weiler et al., 2019). ABS, a commonly used 3D printer filament produces a very rigid product. To avoid this rigidness and aid the ease of unmolding Weiler et al. (2019) used 3D printing to create a negative for a silicone mold, the silicon mold was then cast and used to grow the mycelium. The flexible properties of silicon allowed the MBC to easily release from the mold (Weiler et al., 2019). The use of 3D software allowed the researchers to incorporate textural details, producing an MBC that has more aesthetically pleasing visual features (Weiler et al., 2019).

The growth of mycelium is generational, a small sample of the mother spawn can be taken and used to inoculate a second generation. A small colonized sample from Generation 2 can be taken and used to inoculate generation three and so on, see Figure 3.

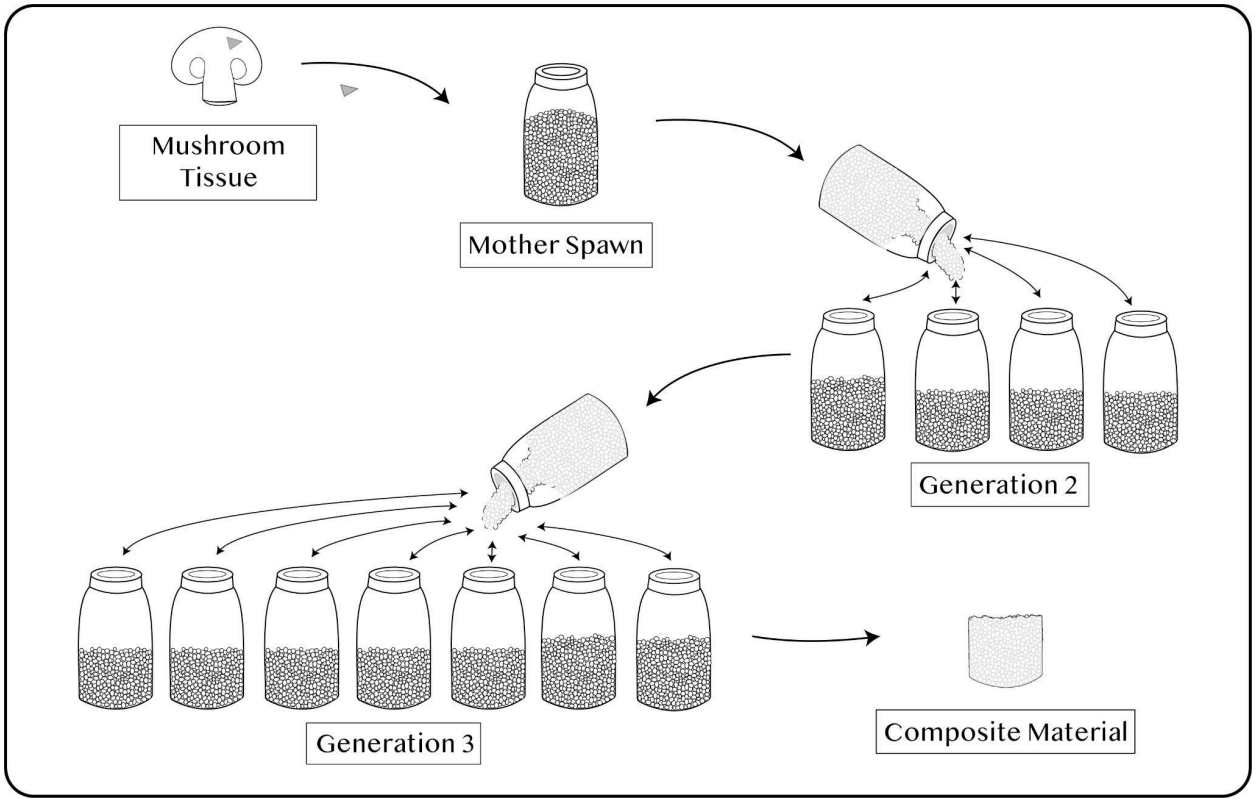

Figure 3 Model of mycelium generational growth.

A lack of information is present in the research showing the entire process of growing mycelium materials with the starting point at a liquid culture to the end of dried material. This allows for difficult replication of the methodology and a gap of understanding in the process variables affecting manufacturing.

2.5 Three Growth Stages

After inoculating the substrate with the mycelium, the mycelium will grow through three phases: the lag phase, the exponential phase, and the stationary phase (Jones et al., 2017). While the mycelium is growing, photo and journal descriptions are taken and used as a method to track the visual assessment of mycelium growth speed and patterns **(**Jones et al., 2017; Lelivelt et al., 2015; & Vasatko et al., 2022). The growth is expected to follow three growth patterns each correlated with a given phase (Lelivelt et al., 2015). The lag phase consists of the mycelium

settling into its new physical and chemical environment, there is little to no growth of mycelium (Jones et al., 2017). The exponential phase coincides with providing the mycelium the optimal growing conditions (Jones et al., 2017). Exponential growth will occur when the mycelium is gaining nutrients from its substrate, growing denser over time (Jones et al., 2017). The stationary phase begins when the mycelium has absorbed most of the nutrients from the substrate and growth will stop (Lelivelt et al., 2015). It is essential to provide optimal growing conditions to lessen the lag phase and encourage dense mycelium during the exponential phase. For Lelivelt et al. (2015), the samples grew for 16 days, while for Vasatko et al. (2022) the samples grew for 20 days, taken out of the mold, flipped, and then grown for five more days. Allowing the mycelium to grow for more days, allowed it to form a denser network and exhibit higher values of compression strength (Vasatko et al., 2022).

2.6 Development of Protocol

The lack of replicable methodology available in the current literature prompted the need for an independent study to learn how to successfully grow the material and identify the process variables that affect the material's composition. A combination of research articles were used to inform the creation of a holistic protocol (Lelivelt et al., 2015, Sharma & Kumar, 2011, & Stamets & Chilton, 1983, 2000). From the literature reviewed, Levlivelt et al., 2015, provided the most thorough protocol for replication. Although, many specifics of the parameters are not included: separation of mother spawn and Generation 2 details on the growing environment and inoculation process, ratios, and specifics for substrate, sterilization, and growing time. This leaves gaps in the research and gray areas of process variables that affect mycelium colonization and the ending material. See Figure 4.

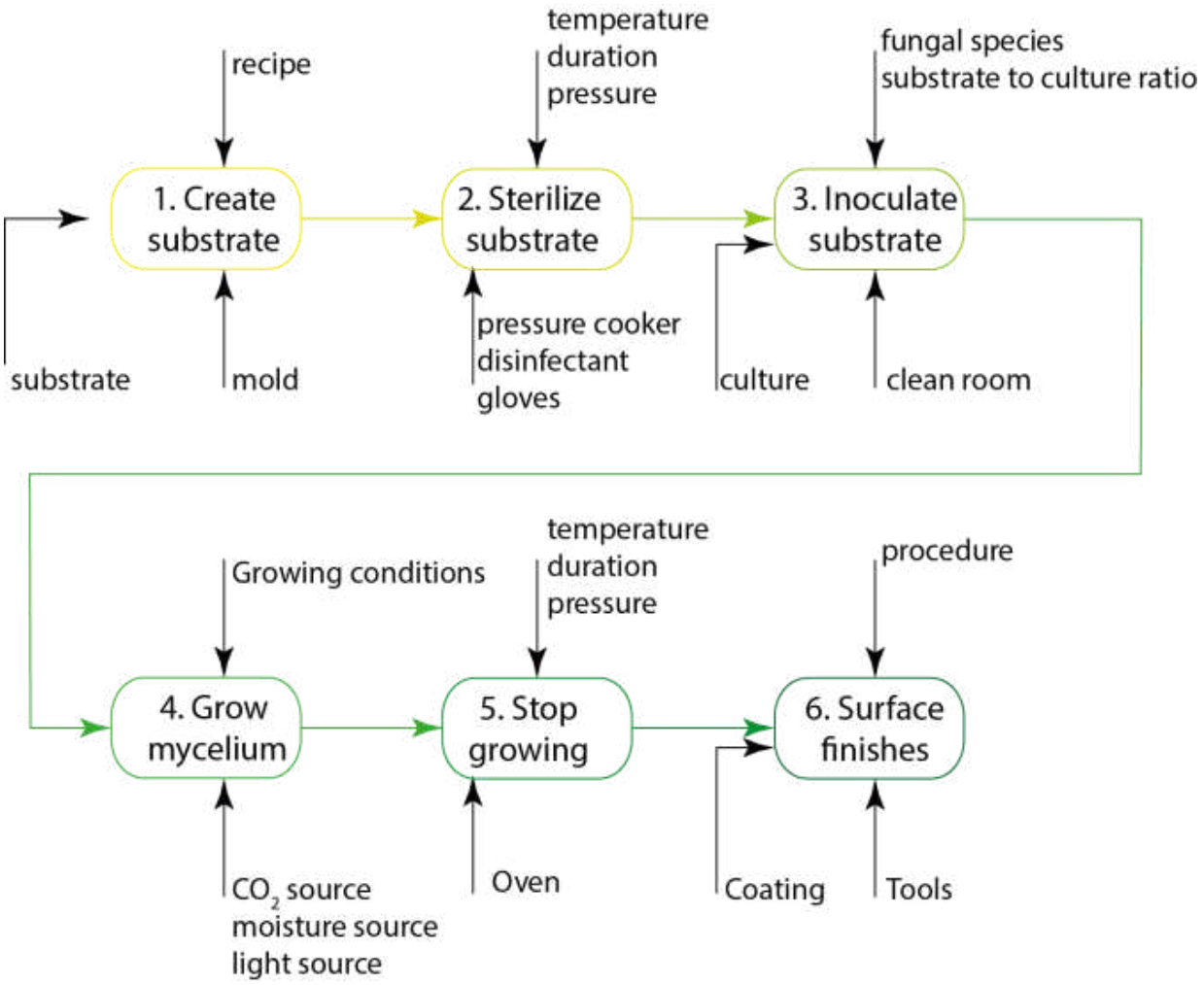

Figure 4 Scheme of the mycelium-based material production process (Lelivelt et al., 2015).

2.7 Motivation for Study

The motivation for this project stems from the published research identifying the increasing problem of environmental pollution, the growing research into mycelium materials as a solution to environmental pollution and an element of bio-design, the lack of replicable protocol, and the need for enhancing performance properties of the composite material (Abhijith et al., 2018; Alemu et al., 2022; Cerimi et al., 2019; Elsacker et al., 2019; Lelivet et al., 2015; Sydor et al., 2022; Vašatko et al., 2022 & Wood, 2019). The current literature suggests the chosen substrates and fungal species as future research for increasing the performance properties

but there are gaps in the research identifying the results of intermixing these substrates together. Cardboard specifically needs further research, suggested by Vašatko et al., 2022, for its excellent compression strength and high density, but was only used in one sample.

As said by Collet, 2017: “What I can not grow, I can not understand”, as a design paradigm shift is happening with the intersection of biology and design, the design approach entails understanding the mechanisms of growth. Beyond understanding how to grow the material, an understanding of what affects the strength of the material is greater. For mycelium materials to reach a wider market value they have to meet the performance standards set by current industry materials. A gap in the literature suggests a need for a holistic understanding of the material, where designers and material scientists can find information relating to the chemical, mechanical, and physical properties of mycelium materials. Implications of this work will shed insight into the way the material's performance can be further developed and material application.

Chapter 3 Material and Experiments

3.1 Overview of the Protocol

The basic structure of the protocol was developed by Lelivelt et al., 2015, but duplicated to identify the separate mother spawn and generation 2 processes. All items with a red background are changes or additions to update Levilet et al., 2015, which identifies gaps in the production parameters and process variables that affect the material's composition, uncovered through literature review and independent study experiments. All items in the yellow box are additions to the protocol made during the independent study, see Figure 5. These additions show how the protocol can be even further developed to identify more manufacturing parameters and reflect gaps in the research related to the chemical, mechanical, and physical properties that are identified in the research hypothesis.

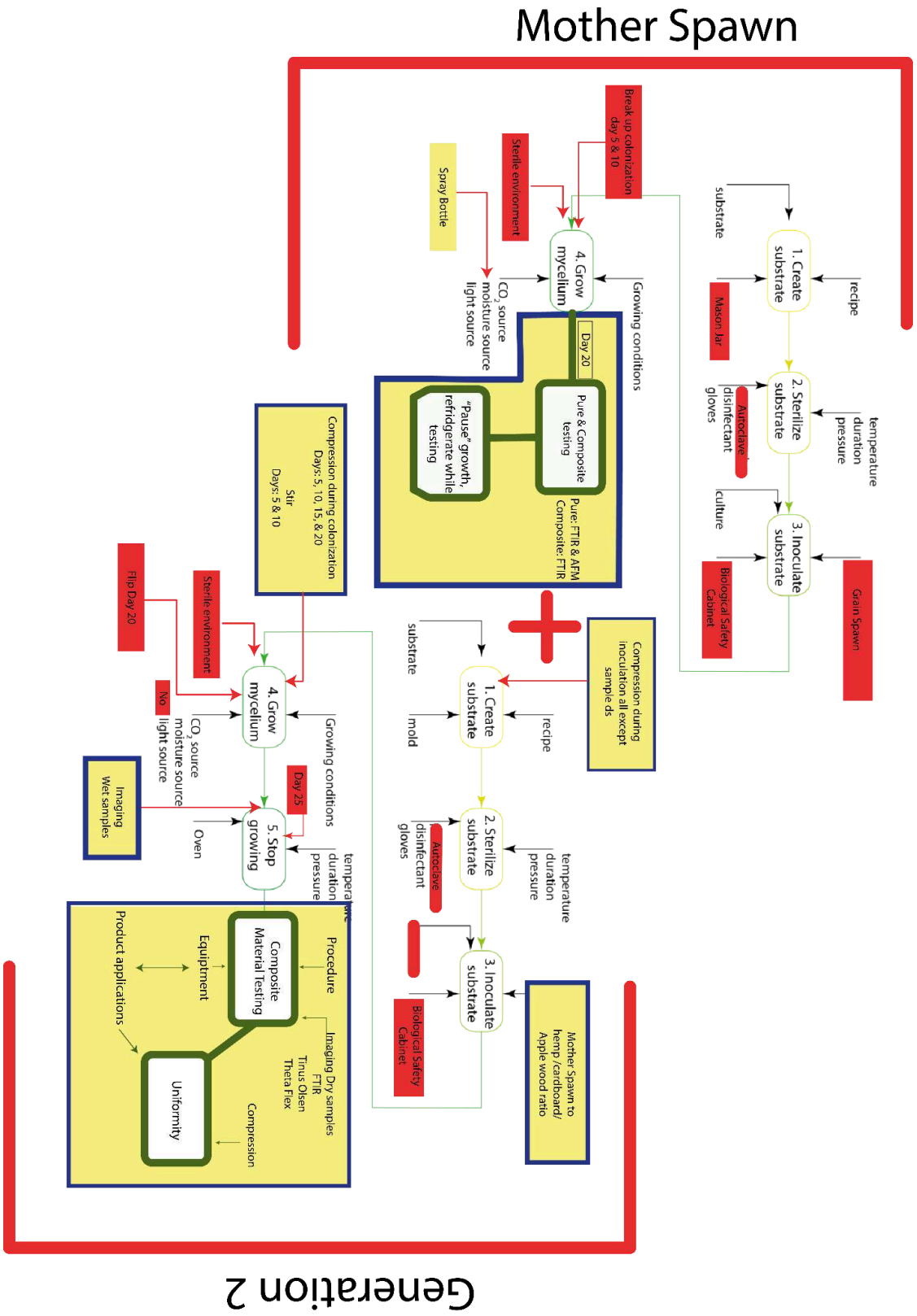

Figure 5 Protocol.

3.2 Preparing Substrates for Mother Spawn and Generation 2

The substrates were prepared with proper sterilization technique 24 hours before inoculation. The mother spawn used rye berries as the nutrient substrate. The berries were soaked for thirty minutes with tap water to allow for the grain to absorb water, then boiled for 25 minutes to allow the grains to be fully hydrated and softened for digestion. The grains were then strained and dried, allowing the excess water to evaporate. Allowing the excess water to evaporate ensures the substrate is not over-saturated with water, which would increase the likelihood of contamination. The growing container was filled ¾ full of grain, covered with tin foil lid, and autoclaved at 22psi at 121 C for 1.5 hours. The jars cool for 24 hours and then were inoculated with the liquid culture.

The substrates for the Generation 2 samples were prepared with proper sterilization technique 24 hours before Generation 2 inoculation. The preparation for these substrates was identical to the mother spawn, except for the nutrient substrates which were cardboard, applewood, and hemp fabric. Each of these substrates were soaked, boiled, strained, and autoclaved in their own containers to reduce contamination. The samples were then move onto the inoculation phase.

3.3 Inoculating Mother Spawn and Generation 2

Inoculation is when the fungal species is introduced and mixed with the nutrient substrate. The fungus species *Pleurotus ostreatus*, was utilized. After 24 hours, when the berries have cooled, the mother spawn was inoculated which will start Day 1 of the growing process. Two samples were made inside the 16 oz mason jar containers that were made during the independent study. The mason jars have two holes at the top. The first hole is covered with Permatex High-Temp Red Silicone and allowed to dry for 24 hours. This acts as a self-healing

port to ensure sterilization while inoculating the commercially sourced liquid culture into the substrate. The second hole is filled with Polyester Fiberfill to act as a filtration system to keep the mycelium environment sterile while also allowing for the flow of oxygen. Before inoculation, the berries were shaken to evenly distribute wet and dry kernels. During the process, proper sterilization techniques was used and work was conducted in the biological safety cabinet. The liquid culture was injected into the self-healing port of the container. Then it will be gently shaken to evenly distribute the mycelium. The jars were stored on a shelf at ambient temperature, at this early stage keeping them in the dark is not as important because the experiment's growth time and container space will not allow for fruiting bodies.

The inoculation of Generation 2 happened after the mother spawn is done colonizing and samples have been taken for data analysis. A similar procedure was be done as in the mother spawns inoculation. Working in the biological safety cabinet with proper sterilization technique the mother spawn will be mixed with different ratios of the nutrient substrates hemp fabric, applewood chips, cardboard, and colonized mother spawn, found below in Table 1. This started Day 1 of the Generation two growth period. Each sample was grown in a 4oz mason jar with a tin foil lid that had holes poked in for oxygen intake. The containers used for growing were 4 oz mason jars. All of the ‘d’ samples are the control and no compression during inoculation or colonization happened. After the samples were made in their containers, an initial compression was done with the 3D printed lid to compact during inoculation, except for d samples.

Table 1 Ratio of Generation 2 samples to nutrient substrates and mother spawn.

Sample ID #	Nutrient Substrates	Weights	Mother Spawn Mycelium
1.1-1.8	Hemp fabric & applewood chips	0.95g fabric, 16g applewood	1 Tbsp

2.1-2.8	Applewood chips	16g applewood	1 Tbsp
3.1-3.8	Hemp fabric & cardboard	0.95g fabric, 16g cardboard	1 Tbsp
4.1-4.8	Cardboard	16g cardboard	1 Tbsp
5.1-5.8	Hemp fabric	0.95g fabric	1 Tbsp
6.1-6.8	Applewood chips & cardboard	8g applewood, 8 g cardboard	1 Tbsp
7.1-7.8	Hemp fabric, applewood chips, & cardboard	0.95g fabric, 8g cardboard, 8g applewood	1 Tbsp

3.4 Growing Parameters and Conditions for Mother Spawn and Generation Two

Humidity and temperature were the main environmental condition to monitor for. The bigger the size of the air intake, the quicker the substrate will dry out. During the mother spawn experiment, after ten days of growing, the colonization began to pick up and the environment was more susceptible to drying out. Air circulation is important, the jars were not packed tightly together and stored away from circulating air. The jars were kept at ambient temperature. On days 5 and 10, the colonization was broken up to encourage thicker colonization. The growing period is 20 days. On day twenty, the mother spawn growth period is over.

During the Generation 2 experiment, the humidity and temperature were also carefully monitored. These samples were kept at ambient temperature and be grown in the dark to prevent fruiting bodies. Compression with a 3D printed device that fits down into the container was done on days 1, 15, & 20 to all samples except d samples. Before each compression, the lid will be sterilized. The growth period for these samples was 25 days. On day 25, the growth period was over and samples were taken for data collection. The samples were removed from their

containers and dried, 195F for 2 hours. The drying prevents the mycelium from further colonization and prevents any fruiting bodies.

3.5 Material Categorization and Analysis

While the mycelium was colonizing for both the mother spawn and generation two, observations will be recorded through journaling and photo documentation. The following aspects were recorded: speed of colonization, areas of colonization, the color of growth, any actions such as watering, stirring, or compression, the temperature of the environment, development of pure mycelium on the surface, and inspection for contamination. At the end of the growth period, the duration of three growth phases was analyzed and identified.

3.5.1 Size and Morphological Characterization

Imaging of the hyphae and its network was done with pure and composite mycelium materials. To avoid potential contamination, all imaging was done with dried samples. After the mother spawn had reached the end of its 20-day growth period, two samples were taken and dried for imaging. This mycelium was not compressed during its growth and allows for the mycelium to branch up, the pure material freely grows up the sides of the mason jar. These samples will be examined using imaging with the Scanning Electron Microscope to analyze the nanostructure of the pure mycelium.

After the 25-day growth period of Generation 2, samples were also taken from the composite materials of each nutrient substrate combination and analyzed. The Scanning Electron Microscope (SEM) was used for imaging and analyzing the surface features of the generation two composite hyphae network. SEM imaging analyzes the formation of the network's structure, diameters, and how each network reacts with the nutrient substrate. Imaging will be compared with results from Haneff et al., 2017 and Silverman et al., 2020 SEM.

3.5.2 Materials Surface Hydrophilicity/Hydrophobicity

Understanding the material's relationship with water is important for insight into product application. The Theta Flex was used to determine the dried materials' contact angle. The Theta Flex requires a uniform surface for accurate recording of the contact angle. With compression during inoculation and colonization, the surface of the material should be more uniform and allow for an accurate reading. The topography of the sample were measured in order to generate a contact angle corrected, which is a more precise measurement of the contact angle.

3.5.3 Chemical and Mechanical Analysis

Understanding the chemical composition of the material leads to connections of the material's strength and better theoretical applications. The Fourier-transform infrared spectroscopy (FTIR) was used in various pure and composite samples. The first sample was taken similarly to the pure sample that is used for SEM. This samples provided a baseline of the chemical composition of the pure mycelium. After Generation 2 samples have finished colonizing, a sample of each nutrient substrate combination were taken, dried, and used for FTIR to understand how each nutrient substrate affects the chemical composition.

The Tinius Olsen in the Gifford Textiles Lab was used for tensile strength testing. The tensile testing was completed on the samples the hemp fabric samples that the mycelium grew on. Additionally, control samples of the hemp fabric without mycleium growth was used to compare how the strength of the fabric was affected based on the mycelium colonization.

3.6 Product Implication

Through conducting an analysis of the mycelium materials' chemical, mechanical, and physical properties, using nutrient substrates that are hypothesized to increase performance

properties, a holistic understanding was gained. This understanding was used to hypothesize product application and make recommendations for future materials experiments.

Chapter 4: Manuscript

The development and characterizations of *Pleurotus fossulatus* mycelium based composite for bio based textile product implications.

Morgan N. Davis[1], Sal Greenberger[2], Jesse Heacock[3], Jane E. Stewart[2], Yan Vivian Li[1234]

1 Department of Design and Merchandising, Colorado State University, Fort Collins, CO.
2 Department of Agricultural Biology, Colorado State University, Fort Collins, CO.
3 School of Biomedical Engineering, Colorado State University, Fort Collins, CO.
4 School of Advanced Materials Discovery, Colorado State University, Fort Collins, CO.

Synopsis

The current materials economy produces linear materials without regard to their end of life. As the demand for these materials rises, the demand for a new textile economy increases: regenerative by design, minimizing resource input, and waste systems. Mycelium-based composites (MBC) is a lightweight biodegradable material, with good thermal insulation, fire resistance, and acoustic attenuation. The role of MBC lies at a crossroads between biology and design, where designers incorporate biological principles and need to understand the mechanisms of material growth. The study's specific aims include the evaluation of the chemical, mechanical, and physical properties of MBC developed in various conditions and nutrient substrates. Essential to this process is the cultivation of the mycelium, where careful considerations of fungal species, nutrient substrate, and growing parameters are critical. A component of the first critical factor emerges: verification of the fungal DNA. The nutrient substrate hemp fabric demonstrates great compatibility, with the fungal species *Pleurotus fossulatus,* as it has the largest hyphae diameter, and offers solutions to incorporate waste. In contrast to the strong hydrophilic nature inherent in many natural cellose, mycelium exhibits hydrophobic properties, a critical feature in terms of product implication. The results provide insight for the future design and optimization of mycelium-based composites for product development innovation.

Keywords: Mycelium, Composite, Sustainability, Bio material

1. Introduction

A new textile economy is reimagined based on principles of Circular Economy (CE), regenerative by design, and provides benefits for business, society, and the environment, nature's circular systems act as a model for the systematic change that is needed[1,2,3]. A systematic change consists of making better use of resources and moving to renewable inputs, where production generates less waste, reduces water use, and requires fewer inputs of nonrenewable resources [1]. Mycelium-based composites have eco-friendly characteristics such as the usage of waste materials, low energy needed during production, the production does not generate waste, and the products are easily recycled [4]. New design practices are developing to incorporate biological principles and tools designed by how nature operates [5].

Bio-design, a design method that incorporates living entities, is an emerging design practice because of its sustainable green principles, offering solutions to global waste [5-7]. The role of mycelium as a material lies at a crossroads between design and biology. This intersection is prompted by a design paradigm shift, where designers are seeking living entities such as mycelium rather than traditional inanimate matter such as plastics and metals [5]. This opens new possibilities for fabricating intelligent materials that focus on new sustainable processes [5]. Mycelium materials have sustainable qualities that aid bio-design by offering alternatives to plastics and solutions to waste [6,8]. Working as a co-designer with mycelium offers solutions to repair and heal the environment.

MBCs have been explored in the packing industry and have proven to be a sustainable replacement for polystyrene [7,9,10]. The production of MBC produces ten times less carbon dioxide (CO2) and about eight times less energy than the production of polystyrene foam [10]. In addition, MBCs are used for other applications such as lamp shades, flower pots, surfboards,

insulation material, and shoe soles [9,11]. MBCs offer value-added through low cost, low emissions, and recyclability [12].

In order to understand the composition of mycelium as a material, it is important to understand how the mycelium grows intertwining itself, resembling a non-woven material [13]. Hyphae are small branch-like strands that form a dense mycelium network [13]. The hyphae network grows and binds together to create a mycelial mat [14]. The feeding fungus acts as a biological glue for the material by colonizing and binding the loose waste together [15]. Using MBC's as a material is a growing body of research [4,7,15].

The composite materials are able to be fine-tuned to control for the structural and mechanical properties, achievable through exploiting different nutrient substrates for hyphae growth, the mechanical properties of mycelium materials are closely related to their nutrient substrates [16]. In addition to the nutrient substrate, growth parameter variation can result in changes in the material mechanical properties [17]. The main factors affecting the production of mycelium composites, and consequently their mechanical behaviors are: mycelium species, substrate, and process variables during manufacturing: protocol, sterilization, inoculation, packing, incubation, growing period, and drying method [17].

It is known that there are three categories of variables affecting the success of growing mycelium. Recent basic research has shown that 1. fungal species, 2. nutrient substrate, and 3. the development of growing parameters (or process variables during manufacturing) are the top critical factor affecting the composition and use of MBC [4,17].

Fungal Species

The Phylum Basidiomycota is typically selected for biomaterial production, specifically because of the mycelium's natural adhesive property and its ability to degrade lignocellulose

[15]. The most commonly used species for mycelium materials that belong to the Phylum Basidiomycota are *Pleurotus ostreatus (*oyster mushroom) and *Ganoderma lucidum (*reishi mushroom*)* [12]. Both are white rot fungi and were found by a basic research study to be the most frequently used species in MBC research [4]. White rot fungi have the capability to excrete a variety of enzymes, some of which are able to degrade plant components difficult to hydrolyze, like lignin in the plant cell wall, compared to brown rot wood degrading fungi that can only degrade cellulose [4,16]. In addition to its frequent use in MBC research, *P. ostreatus* is easily accessible and nontoxic [13, 11,18,19).

Nutrient Substrate

Typically, the substrate material is from three sources: agricultural by-products, industrial waste, or post-consumer waste [4]. These substrates can range from annual plants, to softwood, and hardwoods [4]. Mycelium substrates can also incorporate waste from the food industry, Silverman et al. (2020) demonstrated this by integrating natural textiles and chicken feather waste into their substrate. Using SEM, they demonstrated how mycelium was able to bind to the waste while also feeding itself with the nutrients provided by the waste [11].This offers promise for minimizing consumer waste [7].

Development of Growing Parameters

Manufacturing parameters for MBC include careful consideration of the mycelium growing time and provided conditions. These include inoculation temperature, availability of light, pH and moisture content of the substrate, and material drying method [4]. To avoid contamination, the substrate and any materials used in inoculation need to be properly sterilized. Growing time depends on the dimensions of the mold and the fungal species, ranging from 5 to 42 days, with the optimal temperature ranging from 70°F to 86°F and humidity ranging from

80% to 100% [4]. To form dense mycelium and prevent fruiting bodies mycelium should be grown in the dark [13]. A fungus requires oxygen and produces CO2 during growth, so it is necessary to provide fresh airflow [13]. At the end of its growth, the mycelium is dried which prevents any future growth and turns the mycelium into a material [13].

With an increase in MBC experiments seeking the optimum fungal species for MBC product application, verifying the fungal species via molecular techniques is critical. A study analyzed 284 samples from mushroom spawn preservation centers, companies, and field isolations, and found that 56% of the samples were different species than they had been labeled as when received, emphasizing the need to verify the fungal species for textile product development [20]. Obtaining the DNA identification results allows for more scientific, replicable results and a more impactful contribution to the scientific community, where experiment results can be easily compared and optimal fungal species can be moved forward.

As a design paradigm shift is unfolding with the intersection of biology and design, the design approach entails understanding the mechanisms of growth. Beyond understanding how to grow the material, an understanding of what affects material performance is of greater need. For mycelium materials to reach a wider market value they need to meet or exceed the performance standards set by current industry materials. To increase the performance properties of the material, an understanding and implementation of the nutrient substrates need to be compatible with the chosen fungal species. Previous studies have suggested further research with the nutrient substrates of cardboard, applewood, and natural fibers as reinforcement, as these nutrient substrates have shown high compression strength, colonization density, and minimized waste [11, 18,21]. In the current study, the fungal species *Pleurotus ostreatus* was chosen because it is recommended for future development by many research reports, noting its high compressive

strength, wide availability, and low cost [4, 11, 18]. To achieve a material with high compression strength, compressing the wet material to tightly pack the sample early in the growing stages is suggested, similarly inconsistent pressure from manually filling has been shown to affect the uniformity of the sample in shape and density [11,18]. A novel approach for the development of the growing parameters has been implemented by using a 3D-printed device used during inoculation and colonization.

A gap in the literature suggests a need for a holistic understanding of the material, where designers and material scientists can find information relating to the chemical composition, mechanical, and physical properties of mycelium materials. Thus, the primary goal of this research study is to develop and characterize mycelium-based composites and explore their potential application in biodesign and textile product development. The studys specific aims include the evaluation of the chemical, mechanical, and physical properties of mycelium-based composites developed in various conditions and nutrient substrates. As designers are central to shaping the future of mycelium as a sustainable material, careful selection of critical factors such as fungal species, nutrient substrate, and growing parameters are essential for the development of MBC. A parameter of the first critical factor, fungal identification via molecular techniques emerges. Compression during inoculation and colonization gives the designer the ability to compact the material to make a more uniform shape and composite, although further research is needed for the de molding process. Utilizing waste substrates such as fabric remnants or cardboard enhances MBC circular application by using waste outputs as inputs. The hydropobicity of mycelium is a key feature with significant implications for product development, future research should explore the materials degradation in humid and dry outdoor climates.

2. Materials and Methods

2.1 Materials

Two liquid cultures (mycelium suspended in liquid) of the fungal species *Pleurotus ostreatus* were obtained from Mycelium Emporium (https://www.themyceliumemporium.com/) and Mycosymbiotics (https://mycosymbiotics.com/). The following items were sourced from Amazon (http://amazon.com), applewood chips from the vendor Smokehouse Products, 4 16 oz Mason jars from the vender Global Source group, Polyfil from the vender BUTUZE, Reynolds wrap heavy duty tin foil, Permatex high-temperature silicone, and Rye Berries from the vendor AA Plus. The Amazon shipment box was used for the cardboard. The 7 oz, 100% hemp fabric was sourced from Dharma Trading Company (https://www.dharmatrading.com). 4 oz mason jars were used to grow MBC.

2.2 Methods

Three experiments were conducted to obtain the final composite samples. In the methods, the first step was the fungal species was verified with DNA identification. Secondly, the first generation 'mother spawn' was grown to produce a strong, competitive mycelium ready for inoculation into Generation 2, the third and final experiment. Data collection includes size and morphological characterizations, materials hydrophobicity, and chemical and mechanical characterizations.

2.2.1 Molecular verification of the fungal species

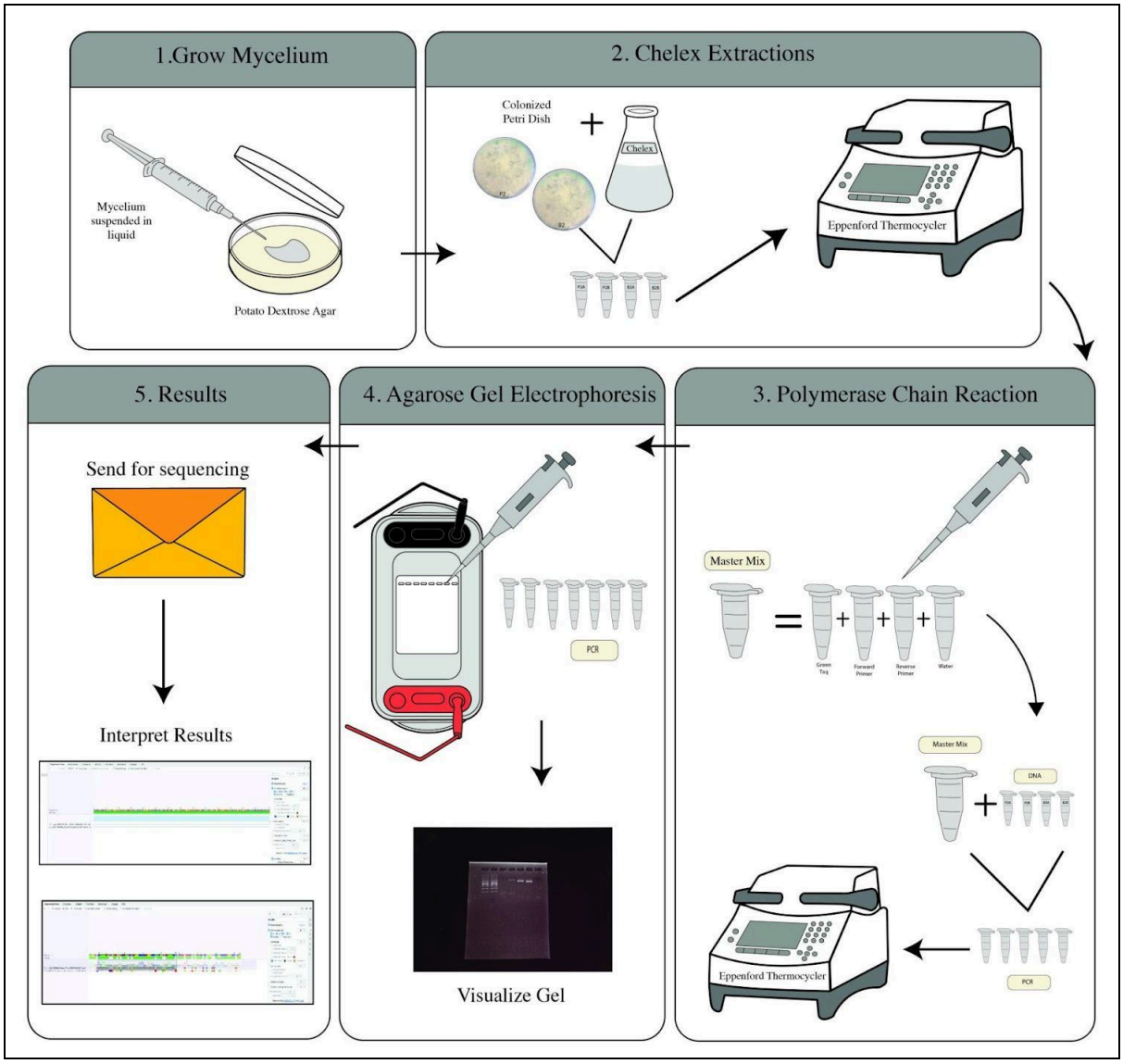

Figure 6: DNA Identification 5-step process, including step 1: grow mycelium. Step 2: Chelex extraction. Step 3: polymerase chain reaction. Step 4: Agarose gel electrophoresis. Step 5: results. Image developed by first author.

Isolates from two liquid cultures (mycelium suspended in solution) were sequenced to verify they were *Pleurotus ostreatus* as advertised. The cultures were sourced from two different affordable commercial companies, each advertised with different common names, “Pearl Oyster" (Mycelium Emporium, Enfield, Main) and “Blue Oyster” (Mycrosymbiotics, Pennsylvania).

We underwent a 5 step process to extract DNA and use molecular approaches to verified the species identification, seen in Figure 6. To begin, 100uL of each liquid culture were pipetted

onto half-strength potato dextrose agar (Hardy Diagnostics; Santa Maria, CA). Plates were incubated at 25°C for 9 days to generate mycelium growth. The genomic DNA was then extracted from the isolates by adding mycelium to 100uL of 5% Chelex® 100 resin (Biorad, Hercules, CA.) solution. Samples were then heated to 98°C for 20 minutes using an Eppendorf Mastercycler PRO 3 Thermal Cycler (Enfield, CT.) Polymerase chain reaction (PCR), was then used to amplify specific regions of DNA, for sequencing and genetic analysis. PCR protocols were adapted and are as follows [22]. The partial translation elongation factor 1-α (*TEF*1) gene was amplified using the primer pair 728F and 1567R [23,24]. PCR was performed with an Eppendorf Mastercycler PRO Thermal Cycler (Enfield, CT) using a 20-µl reaction mixture comprising 1 µl template DNA, 7 µl of autoclaved molecular grade water, 1 µl of each primer, and 10 µl GoTaq® Green Master Mix (Promega Corporation, Madison, WI.) The PCR cycle included 1 cycle of 5 minutes at 94°C, 10 cycles of 30s at 94°C, 55s at 63°C, and 90s at 72°C. Plus 36 cycles of 30s at 94°C, 55s at 53°C, and 90s at 72°, followed by one cycle of 7 minutes at 72°C and a perpetual hold at 4°C.

PCR generates millions of DNA fragments, to separate the fragments and analyze the bands by size, quantity, and quality, an agarose gel electrophoresis was run as the fourth step. PCR products were visualized by running a 1.5% agarose gel with 0.5X TBE buffer and stained with GelRed™ Nucleic Acid Gel Stain (Gold Biotechnology, St. Louis, MO.) UV light was used to confirm amplification. Finally, successful PCR products were purified using ExoSAP-IT® PCR Product Cleanup (appliedbiosystems by Thermo Fisher, Vilnus Lithuania) following manufacturer's instructions and were Sanger sequenced at Eurofins (MWG Operon USA, Louisville, KY.)

2.2.2 Mother Spawn

To prepare the nutrient substrate for the mother spawn, rye berries were soaked, boiled, drained, and allowed to dry for one hour. Next, the rye berries were put in the 16 oz mason jars filled to ¾ full and autoclaved at 121° C for 1 hour. The jars cooled for twenty-four hours. Working in a biological safety cabinet, the liquid cultures were inoculated onto the substrate through the injection port on the lid. The mother spawn samples were grown for 22 days prior to inoculating Generation 2. During the 22-day growth period, qualitative observations were recorded, including the colonization patterns, color, growth rate, and apparent moisture of the samples. To consume less energy, the samples were grown in ambient temperature, therefore, the temperature of the room was recorded each day, and ranged from 63.8° F and average to 67.35°F. The mycelium grew through three phases: the lag phase, the exponential phase, and the stationary phase [25]. The growth is expected to follow three growth patterns each correlated with a given phase [13]. The lag phase consists of the mycelium settling into its new physical and chemical environment, there is little to no growth of mycelium [25]. The exponential phase coincides with providing the mycelium the optimal growing conditions [25]. Exponential growth will occur when the mycelium is gaining nutrients from its substrate, growing denser over time [25]. The stationary phase begins when the mycelium has absorbed most of the nutrients from the substrate and growth will stop [13]. The lag phases lasted days 1-8, the exponential phase lasted days 8-21, and the stationary phase lasted days 21-22. Before the mother spawn was used for inoculation of Generation 2, some pieces of the mycelium was scraped off the side of the jar, where it had grown up and branched off the composite base and was dried for 2 hours at 195°F for the pure samples.

Figure 7: Left: Removal of pure material, Right: substrate material for Generation 2

2.2.3 Generation Two

To prepare the nutrient substrates for Generation 2, the hemp fabric was cut in a circular shape with a diameter of 2 inches. It was then soaked for 30 minutes in tap water, the excess water was squeezed and allowed to sit for an hour in order to not be overly wet. The cardboard box was inspected and pieces without ink or stickers present were cut down into approximately 2 x 2-inch squares, then torn and mixed in a blender which resulted in a variety of sizes ranging from 1.25 inches or less. It was sprayed with water to get damp and sat for an hour. The smaller-size applewood chips were chosen from the pre-cut bag and ranged in length from 1.75 inches or less. They were soaked for thirty minutes in tap water, boiled, drained, and let dry for an hour. After the nutrients were prepared, each sample was prepared using different ratios of hemp fabric, cardboard, and applewood chips in a 4 oz mason jar and autoclave at 121 C° for one hour (Table 2). The jars cooled for 24 hours and were moved into the Biological Safety Cabinet.

Table 2: The sample ID #, and ratios of Generation 2 samples to nutrient substrates and mother spawn mycelium.

Sample ID #	Nutrient Substrates	Weights	Mother Spawn Mycelium
1.1-1.8	Hemp fabric & applewood chips	0.95g fabric, 16g applewood	1 Tbsp
2.1-2.8	Applewood chips	16g applewood	1 Tbsp
3.1-3.8	Hemp fabric & cardboard	0.95g fabric, 16g cardboard	1 Tbsp
4.1-4.8	Cardboard	16g cardboard	1 Tbsp
5.1-5.8	Hemp fabric	0.95g fabric	1 Tbsp
6.1-6.8	Applewood chips & cardboard	8g applewood, 8 g cardboard	1 Tbsp
7.1-7.8	Hemp fabric, applewood chips, & cardboard	0.95g fabric, 8g cardboard, 8g applewood	1 Tbsp

After cooling, the samples were inoculated with one tablespoon of the mother spawn mycelium per jar. The samples were mixed and then compressed with a 3D printed device, the samples ending in .8 were not compressed as a control (Table 2). The samples were grown in the dark for 25 days, compression for the appropriate samples took place on days 1, 15, and 24. The samples were dried in their 4 oz mason jars at 195°F for 2 hours.

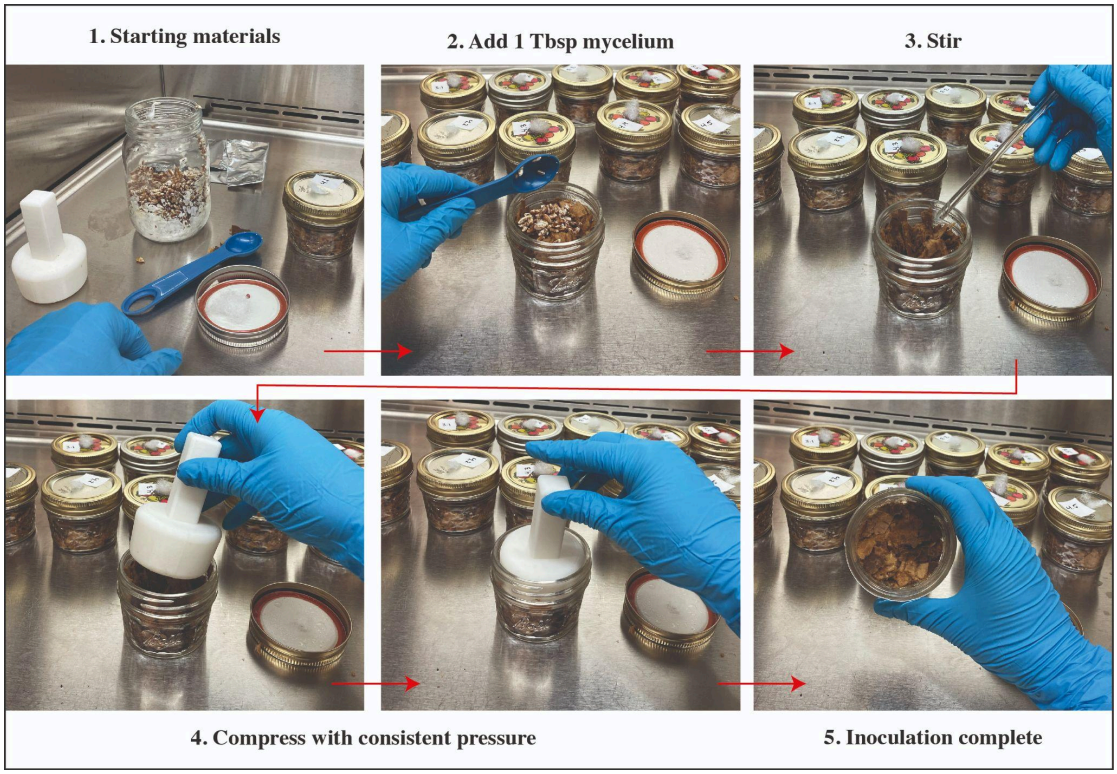

Figure 8: Use of 3D printed device in inoculation including step 1: starting materials. Step 2: add 1 tablespoon of mycelium. Step 3: stir. Step 4: use device to compress with consistent pressure. Step 5: close jar and inoculation complete.

2.2.4 Size and Morphological Characterization

The size and morphology of fibers were investigated using a scanning electron microscope (JEOL IT700HR), for both pure mycelium and mycelium composite materials. Pure samples were obtained from the 22-day-old mother spawn, characterized by mycelium that had branched up, off the base of the composite, and onto the sides of the growing container and then dried. Composite samples are dried Generation 2 samples. The composites were carefully broken apart to obtain small pieces, double stick carbon tape was used to adhere to the sample holder. Then the samples were sputter-coated with gold to improve conductivity. After the images were obtained, Image J Software was used to collect the mean and standard deviation of the hyphae diameter for each substrate.

2.2.5 Materials Hydrophobicity

An Optical Tensiometer Theta Flex (Biolin Scientific with 3D topography unit) was used to characterize the materials hydrophobicity of pure mycelium, composite mycelium (mycelium and hemp fabric, mycelium and applewood chips, and mycelium and cardboard), and substrates without mycelium (hemp fabric, applewood chip, and cardboard). The composite samples were broken into small pieces, where the water droplet landed directly on mycelium with no substrate. Three repeat samples were collected to average the contact angle and the contact angle corrected. The contact angle corrected takes the material's topography into consideration. A material with a contact angle greater than 90 is considered hydrophobic and will repel water.

2.2.6 Chemical and Mechanical Characterization

The Fourier-transform Infrared Spectroscopy (FTIR Cary 630, Agilent Technologies) was used to characterize the chemical nature of both pure mycelium and composite mycelium: mycelium and hemp fabric, mycelium and applewood chips, and mycelium and cardboard. For the composite samples, the mycelium was pulled off its substrate for a sample that is 100% mycelium with no visible nutrient substrate pieces. Mycelium on fabric was the most difficult to obtain with no visible fabric, as the mycelium was heavily integrated in the fibers. All spectrums were recorded in the range of 4000 to 600 cm^{-1}, accumulating in 128 scans. To ensure the reproducibility of the spectra obtained, three samples of each type were measured.

The tensile strength of the mycelium grown on hemp fabric was measured (Tinius Olsen H5KL with HT400 pneumatic grip control) with adapted methods according to ASTM D 5034-08. The fabric circles were sized to fit the bottom of the 4oz mason jars and measured 2 inches in diameter. The sample size and gauge length were adapted from the ASTM standard to fit the size of the samples, the sizing poses a limitation as the strength of the fabric as a whole

can not be recorded, but rather a smaller piece. According to the standard eight samples were recorded in the filling direction and five samples were recorded in the machine direction. From the mycelium on hemp fabric samples, seven samples were tested in the machine direction, two were removed because the sample slipped or tore too close to the grips, leaving five averaged samples. One limitation prior to growing is that the warp or weft direction of the fabric was not recorded on the mycelium samples, therefore, it was unclear what direction the samples were recorded, and all samples were inputted as machine direction into the software. The mycelium grown on hemp fabric did not bind to the bottom of the growing container and was not structurally damaged when removed.

3. Results and Discussion

3.1 Molecular Species Identification

Sequences of the EF region obtained from Sanger sequencing were compared to the National Center for Biotechnology Information database using the Blast algorithm. The results are as follows, the “Pearl Oyster " sourced from Mycelium Emporium, was *Pleurotus fossulatus*. The “Blue Oyster", from Mycrosymbiotics, was a mixture of several species. The remaining liquid culture for *Pleurotus fossulatus* was used for the mother spawn and Generation 2 experiments. The discrepancy between the advertised species and the actual species identified highlights potential issues with the accuracy of commercial fungal cultures. This raises concern for researchers and cultivators who rely on these for their work. The misidentification can lead to unintended inaccuracies in experimental results. The Blue Oyster culture containing a mixture of several species indicated potential contamination or lack of purity which can significantly impact research outcomes. These findings emphasize the critical role of molecular identification techniques in confirming the identity and purity of fungal cultures. Future work should prioritize

the verification process to ensure the reliability and accuracy of their work. Additionally, this issue calls for increased transparency and accountability within the commercial fungal culture industries: to reduce the risk of misidentification and ensure the integrity of scientific research and cultivation practices.

3.2 Growing Parameters and Compression

3.2.1 Development of Growing Parameters

During colonization, qualitative assessments and temperature were recorded, with the average low at 66°F and average high at 67.25°F. The lag phase was much shorter, lasting until day 4, the exponential phase was between days 4-24, with the stationary phase lasting from days 24-25. It is essential to provide optimal growing conditions to lessen the lag phase and encourage dense mycelium during the exponential phase. The lag phase was 8 days for the mother spawn and considerably less (4 days) for Generation 2. The lag phase lasted longer than expected for the mother spawn and is hypothesized that the temperature of the room or the ratio of mycelium to substrate might have affected the mycelium initial colonization rate. Images of the final Generation 2 samples are in Figure 9. The use of glass mason jars kept the humidity ideal for growth, and was easy to monitor the growth and contamination. The mother spawn was mixed once during colonization to spread the mycelium and generate denser colonization. The longer the mycelium grew, the more denser the network formed [18].

Figure 9: Top and bottom images of the dried composite samples. From left to right, 1. applewood and hemp fabric, 2. applewood, 3. cardboard and hemp fabric, 4. cardboard, 5. hemp fabric, 6. applewood and cardboard, and 7. applewood cardboard and hemp fabric.

3.2.2 Effect of 3D Printed Device

A 3D printed device was created to fit into the 4 oz mason jar growing containers. As suggested for further development, to account for the inconsistent pressure from manually filling and to achieve a material with a more uniform surface, periodic compression was used during inoculation (day 1) and colonization (day 15 and 24) for the Generation 2 samples [14,18]. Since mycelium is a shapeable material, it grew to the shape of the jar, except the top where there was a gap between the surface of the composite and the lid of the jar, therefore it was able to freely grow up and on the sides of the jar. The use of the 3D printed device created a more uniform surface where the top of the composite was flattened and had a similar shape to the bottom of the composite. The device compacted the material, allowing for a more sturdy composite. Although, the samples that underwent periodic compression adhered to the bottom of the jar and binded to the glass, see in Figure 10, where the mycelium penetrated through the fabric and bound to the glass during the unmolding process. Removing the samples from the jar presented issues and the stability of the samples were affected, therefore mechanical testing of compression couldn't be done because the samples integrity was damaged from the unmolding process.

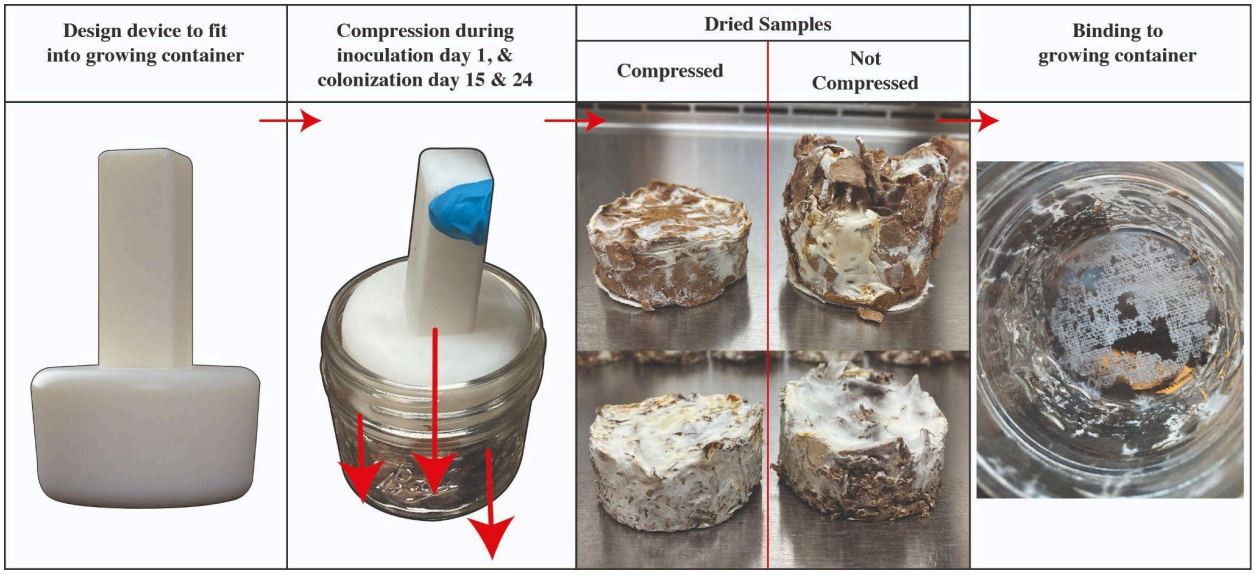

Figure 10: Design and implementation of 3D printed device. Dried composite samples demonstrating difference in sample uniformity with/without compression. Binding of mycelium to container after demolding sample.

3.3 Size and Morphological Characterization

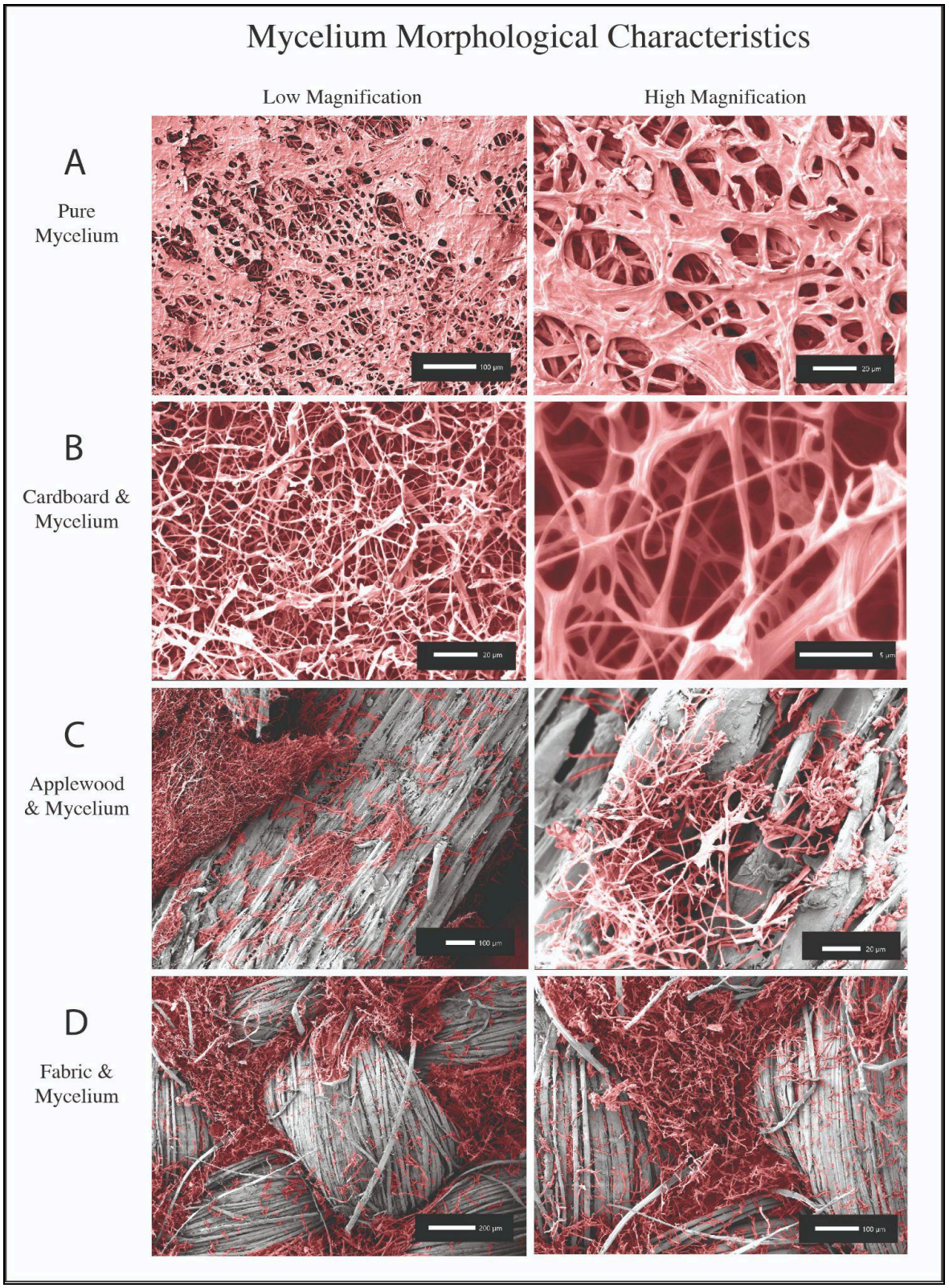

Figure 11: Scanning Electron Microscope with mycelium colored red to show interaction. A. pure mycelium, B. cardboard and mycelium, C. applewood and mycelium, D. hemp fabric and mycleium.

The Scanning Electron Microscope (SEM) imaging of mycelium growth and network interactions between the mycelium hyphae and nutrient substrate are shown in Figure 11. Previous research demonstrated how SEM can be used to observe the mycelium network and observe the relationship between the network and the substrate [11,16]. After the images were obtained, the mycelium was colored red using Adobe Photoshop, to defines the mycelium (red) versus its nutrient substrate (grayscale) and indicates a clear interaction between the two, confirming the mycelium's ability to grow along the substrates under the experiment conditions. Mycelium grows through a symbiotic relationship with the materials that it is being fed, forming a dense network of fibers [16]. The pure mycelium (Figure 11A), is colored completely red because it is made from mycelium that has branched off of the substrate and up onto the side of its growing container, effectively attaching to the side of the container. The cardboard and mycelium (Figure 11B) is also colored completely red because it is unclear how much of a difference between the fibers, perhaps the mycelium has partially or completely consumed the cardboard and has fused together. The applewood and mycelium (Figure 11C) have a rich interaction, binding to and throughout the wood chips, since the fungal species is a wood degrading species, this interaction is to be expected. The hemp fabric and mycelium (Figure 11D) have a similar interaction, where the hyphae are binding between and through the hemp fibers. It is clear that the mycelium penetrated the fabric, firmly attaching and using the hemp fabric as nutrients for colonization.

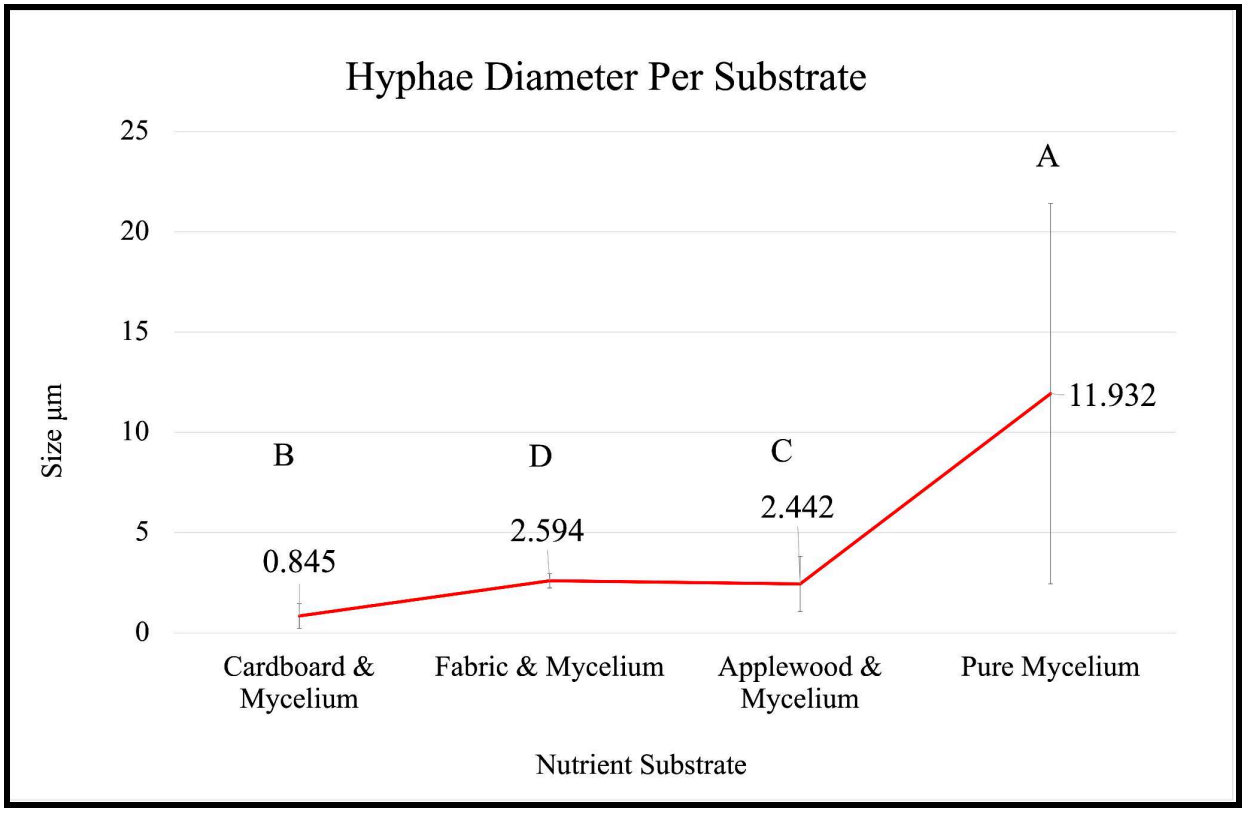

Figure 12: Hyphae diameter average per substrate A. pure mycelium. B. cardboard and mycelium. C. applewood and mycelium. D. hemp fabric and mycelium.

The width of the hyphae depends on the feeding substrates [16]. Typical diameters of hyphea are reported in literature between 1 and 30µm [26, 27]. However, Escaleira et al., (2020) found for the hyphae of *P. ostreatus, G. lucidum* and *H. ulmarius* that grew on pinewood substrates, the diameters were approximately .2µm [28]. While all of the diameters reported in this study lies within 1-30µm, the diameters for each substrate were noteably larger than .2µm. The mean and standard deviation of the fiber diameters are found in Figure 12. The pure fibers (Figure 12A) had the largest mean of fiber diameter at 11.932µm with a standard deviation of 9.248µm, compared to a mean of 2.594µm on hemp fabric (Figure 12D) with a standard deviation of .344µm, 2.442µm on applewood chips (Figure 12C) with a standard deviation of 1.371µm, and .845µm on cardboard (Figure 12B) with a standard deviation of .612µm. The pure fibers had the widest array of range in diameter as the morphology is quite different from the

composite samples, due to the mycelium branching up off the base of the mother spawn and onto the walls of the container, the morphology shows scaffolds that likely formed to support the vertical growth of the mycelium. The average length between the scaffolds in the pure fibers (A) is 10.627µm with a standard deviation of 6.881µm. To compare the size of the mycelium hyphae to the hemp fiber, measurements were taken on the hemp fibers with an average diameter of 15.578µm with a standard deviation of 3.163µm. As the fungal species is a wood degrading, it was hypothesized the diameter would be the largest for Figure 12C, the applewood substrate, although Figure 12D, the hemp fabric substrate diameter is slightly larger with a smaller standard deviation. The compatibility with the hemp fabric points to future research with this material. This suggests that the fungal species used nutrients from the fabric as energy to colonize, grow a larger hyphae, and may have a particular liking for the composition of hemp, which could open up new possibilities for incorporating fabric remnants or scraps.

3.4 Materials Hydrophobicity

Previous research demonstrates the hydrophobicity of mycelium materials with contact angles greater than 90°[16, 29]. Although, this study includes the incorporation of topography in the measurement of the contact angle to produce a corrected contact angle. The corrected contact angle generates a more precise measurement. The report generated for each sample includes an image of the sample, its 2D topography, 3D topography, and the water droplet on the sample shown in Figure 13. The scale bar on the side of the 3D topography represents the difference in the surface roughness, with the red hues are + 30µm and blue hues are -30µm. The mycelium surface is not uniform in roughness, due to the fungal self growing abilities, as shown in the Figure 13 2D and 3D topography images, emphasizing the need for the corrected contact angle,

where the topography of the surface is taken into consideration for calculating the corrected contact angle.

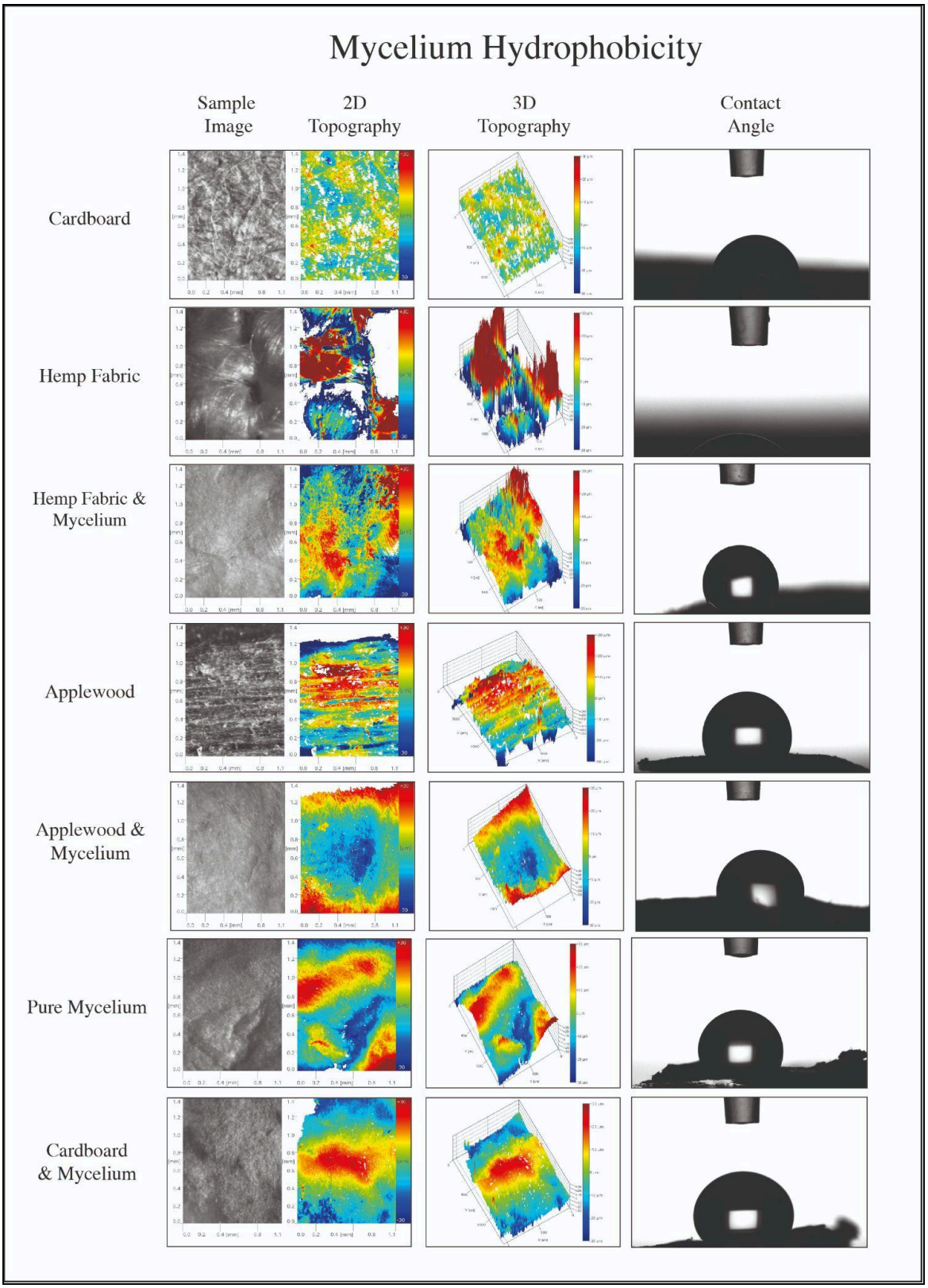

Figure 13: Image of sample, 2D, 3D topography, and water drop images. Demonstrating hydrophobicity/hydrophilicity of mycelium composite, pure, and substrate materials.

Figure 14 demonstrates the contact angle and contact angle corrected for each sample. A hydrophobic material has a contact angle 90°or higher, whereby 90° has been emphasized with a red line on Figure 14. Besides wood, a naturally hydrophobic material, the samples with mycelium increased its hydrophobicity determined by their contact angle corrected: pure mycelium at 99.39°, mycelium on hemp fabric 90.75° compared to hemp fabric only 85.86°, mycelium on applewood 95.17° compared to applewood only 94.49°, and mycelium on cardboard 98.31° compared to cardboard only 83.58°. Naturally occuring cellulose are typically hydrophilic, which can limit their product applicability and strengthens the need for synthetic polymers. The hydrophobic nature of mycelium has been attributed to the assemblies of surface-active proteins, hydrophobin [29]. In addition to the hydrophobins, another consideration is that the unique 3D structure of the mycelium on a micron scale may contribute to the hydrophobicity of the mycelium materials. The hydrophobic nature of the mycelium materials offers a range of benefits that can potentially increase product performance, longevity, and functionality across various industries and applications.

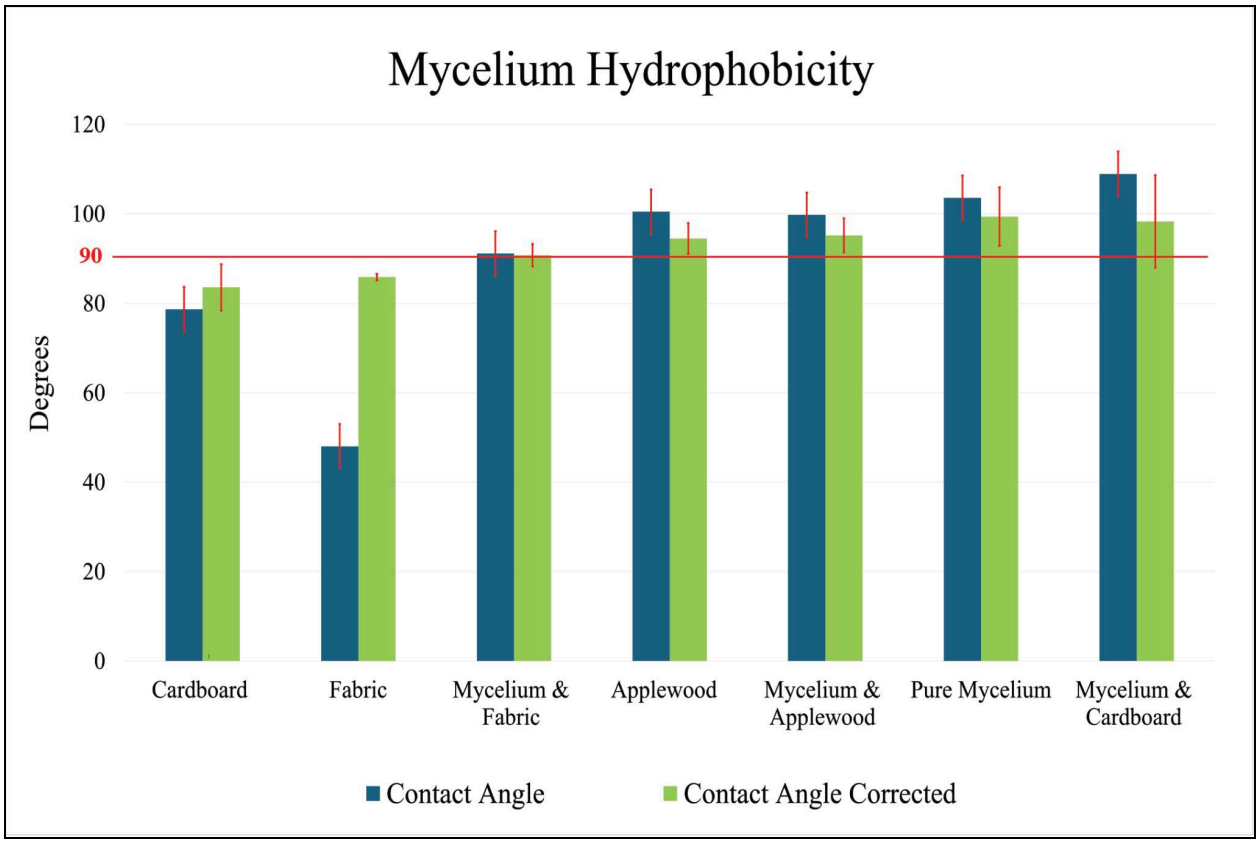

Figure 14: Bar chart demonstrating mycelium contact angle and contact angle corrected. Red line at 90° demonstrating added hydrophobicity of mycelium.

3.5 Chemical and Mechanical Analysis

3.5.1 Chemical Analysis

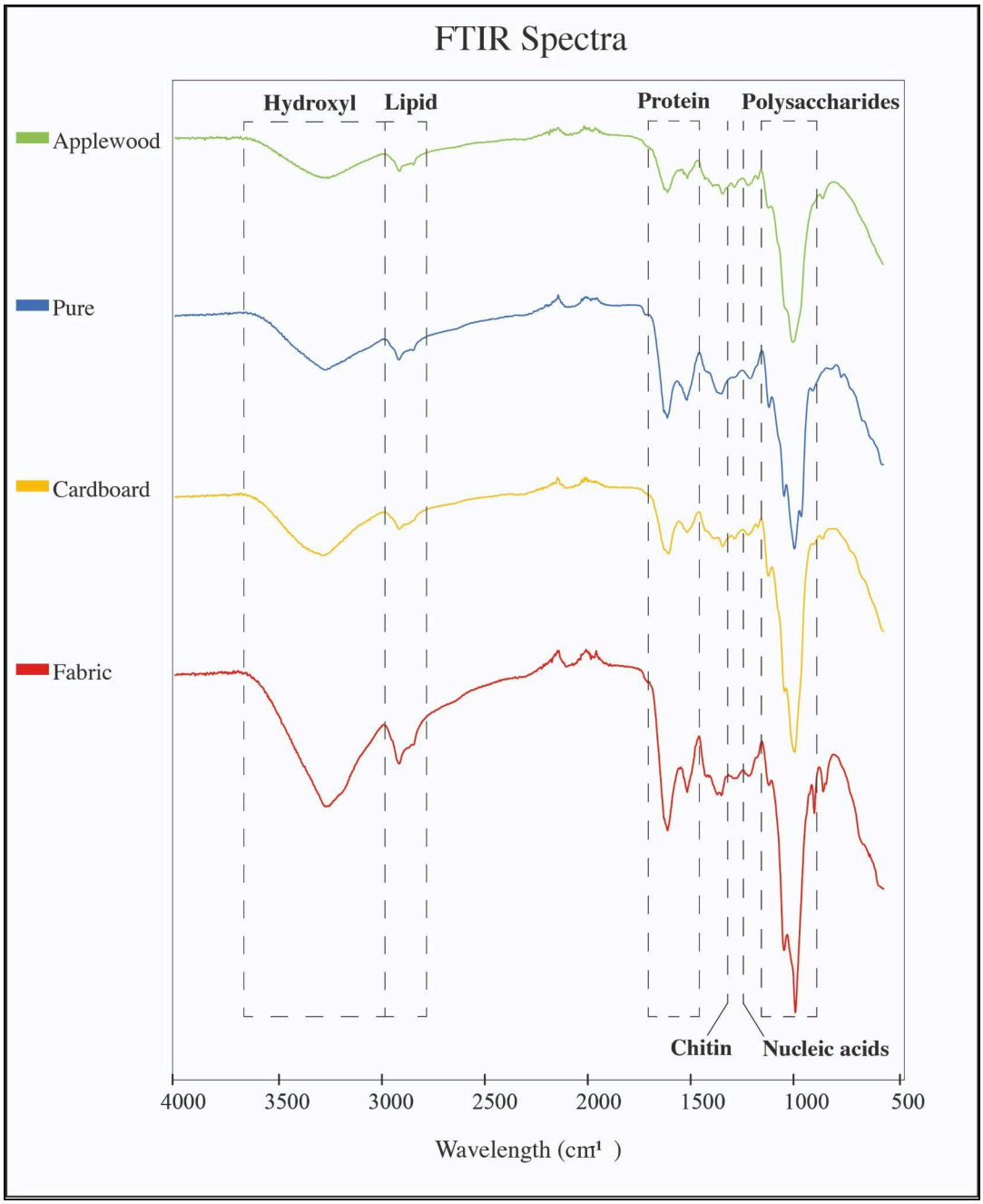

Figure 15: FTIR spectra of mycelium from pure mycelium and applewood and mycelium, cardboard and mycelium, and hemp fabric and mycelium composite samples.

FTIR spectra of the four different types of mycelium are shown in Figure 15. The infrared absorption spectra of the mycelia are associated with the functional groups and biomolecules that compose them, hydroxyl group (3600-3200 cm^{-1}) lipids (3000-2800 cm^{-1}), proteins specifically amide Ⅰ and Ⅱ (1700-1600 cm^{-1}), chitin (1334-1353 cm^{-1}), nucleic acids

(1255-1245 cm^{-1}), and polysaccharides (1200-900 cm^{-1}) [16, 30] In general, the peaks for each substrate align with each other, demonstrating no drastic chemical difference per difference in feeding material. Since the chemical composition of each sample did not drastically change, this indicates that the chemical composition will not affect the mechanical or physical properties of the mycelium for these nutrient substrates.

3.5.2 Mechanical Analysis

The hemp fabric samples stretched in the machine direction had a tensile modulus of 1.32MPa, while the filling direction samples had a tensile modulus of .49MPa. The mycelium on hemp fabric samples had a tensile modulus of .02 MPA. The mycelium on hemp fabric tensile modulus was significantly lower than the machine and filling samples. Suggesting that the mycelium lessens the strength of the fabric. Perhaps the strength is less because the mycelium it is breaking down the fibers and using it as nutrients to grow. As seen in the SEM, the mycelium penetrates through and around the fabric. While the tensile strength did not increase, the mycelium consumed the hemp fiber as nutrients to colonize. Future work should consider further mechanical testing such as compression strength.

3.6 Product Implication

The hydrophobicity of the mycelium is a critical feature of mycelium materials, particularly in terms of product implication. In contrast to the strong hydrophilic nature inherent in many natural polymers, like cellulose, which strongly limits their market application, mycelium exhibits a distinct advantage, its hydrophobic nature. This characterization compares to conventional synthetic polymers, which are widely used due to their resistance to water. Compared to synthetic polymers, mycelium's superiority is highlighted because of its added sustainable production methods. The ability to repel water makes mycelium materials well-suited

for products that need to withstand exposure to moisture or damp environments. This feature will help to prevent damage caused by water absorption such as warping, swelling, or bacterial growth. The added durability in humid or wet conditions poses questions into the materials future in outdoor applications. The hydrophobic nature of the mycelium materials offers a range of benefits that can enhance product performance, longevity, and functionality across various industries and applications. Future research should prioritize degradation studies to assess the materials resilience material in moist or outdoor environments.

Researchers have been investigating nanotechnology through the lens of biomimicry, drawing inspiration from natural phenomena such as a lotus leaf's natural hydrophobicity and self cleaning abilities [31]. The hydrophobic nature of mycelium material is likely due to its 3D structure on a nanoscale, similar to a lotus leaf, which has a contact angle greater than 150°. This unique structure enables droplets to roll off the surface carrying away any debris that the leaf contains, signifying natural self-cleaning properties [31]. The nanoscale of the mycelium 3D structure perhaps contributes to its natural hydrophobicity and holds promise for further exploration for biomimicry in coating different materials for outdoor applications. However, further investigation is needed into the self cleaning properties of mycelium materials.

The successful growth on cardboard and hemp fabric illustrates the potential of utilizing waste as a nutrient substrate for the material, as demonstrated through SEM imaging and dense visible colonization. This enhances the materials applicability within a circular economy framework, where wastes are inputs are repurposed and utilized efficiently. The versatility to be molded into various shapes and forms, allows for vast potential for product development. This not only allows for precise customization but also minimizes waste material scraps, aligning with the principles of a circular economy.

Although compacting the material during inoculation and colonization with a 3D printed device yielded a sturdier and more uniform material, as demonstrated in Figure 10 dried samples compressed vs non compressed, further investigations into the demolding process are needed due to the binding of mycelium to the bottom of the container. Future research should prioritize collecting data on compression strength as this mechanical aspect can significantly add value to the materials performance properties, especially when comparing to materials that are currently used.

4. Conclusions

The exploration of mycelium materials represents a significant opportunity for sustainable and innovative solutions. At the intersection of design and biology, mycelium materials offer a unique collaboration for product designers to work with living entities through the materials entire life cycle, from conception to final product. This paradigm shift highlights the pivotal role of designers in creating intelligent biological materials. Essential to this process is the cultivation of the mycelium, where careful considerations of fungal species, nutrient substrate, and growing parameters are critical. In addition to these factors, a component of the first critical factor emerges: verification of the fungal DNA. This component ensures the reliability and replicability of MBC research studies. These factors directly influence the end properties of the material, highlighting the importance of careful and knowledgeable cultivation practices for the designer. As the potential of mycelium materials continues to unfold, it becomes clear that designers play a central role in shaping the future of sustainable materials. By embracing the design paradigm shift, we pave the way for the development of innovative solutions that not only meet the needs of today but also contribute to a more sustainable and resilient future.

Author Contributions

Conceptualization, M.D., Y.V.L, and J.S.; methodology, M.D., S.G. and J.H.; validation, Y.V.L., J.H., and S.G.; formal analysis, M.D. and S.G.; investigation, M.D and J.H.; resources, Y.V.L. and J.S.; data curation, M.D.; writing—original draft preparation, M.D.; writing—review and editing, Y.V.L., and S.G.; visualization, M.D.; supervision, Y.V.L.; project administration, M.D.; funding acquisition, M.D. and Y.V.L. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 5. Conclusion

This development and characterization of mycelium based composites lays the groundwork for future product exploration studies. The urgent need to address waste generation and environmental degradation from the generation of linear materials prompts a design paradigm shift. This shift occurs at the intersection of design and biology where designers are cultivators of living entities to create intelligent eco-friendly materials. This shift aligns with the principle of the theoretical framework of Circular economy, a regenerative system that minimizes resource input and waste systems by narrowing and closing materials and energy loops. Circular materials provide benefits to businesses, society, and the environment.

The study emphasizes the importances of ensuring compatibility among critical factors in developing MBC, such as the fungal species, nutrient substrate, and growing parameters. These factors play a crucial role in shaping the properties and performance of mycelium materials. In addition to these factors, DNA verification emerges as a crucial component of the first critical factor for the future of mycelium material studies. This extra step adds additional accuracy and reliability of the research.

The hydrophobicity of mycelium materials provides a unique quality for product implication. Cellulose materials generally exhibit strong hydrophilic properties, while mycelium materials is the opposite and repels water. This characteristic should be further explored in applications that require resistance to moisture or exposure to damp environments. The hydrophobicity opens up possibilities for outdoor applications. The durability and degradation time of this material is relatively unknown and prompts the question: How can these materials be durable but also biodegradable? As sustainable materials become more essential, the hydrophobicity of mycelium materials offers a unique advantage over other natural polymers.

The unique 3D structure of mycelium materials also offers an interesting component where biomimicry principles could be further studied.

Future direction for mycelium materials should focus on development of growing parameters, particularly utilizing compression during the growing process. 3D printing gives the designer flexibility to make customized, shapeable devices adapted to the mycelium growing container. Attention should be given to the de-molding process, to prevent the mycelium from binding to the bottom of the growing container. This factor is crucial for maintaining the structural integrity of the material and will allow the researchers to perform further mechanical testing such as compression strength. Understanding the compression strength will provide valuable insights for potential product applications, as it will inform the designer about the materials ability to withstand loads. By addressing these considerations, future research can discover new possibilities for mycelium products and contribute to the advancement of this sustainable material.

As with any research, the process of increasing the performance properties of mycelium materials is a collaboration and the integration of insights gained from existing and future studies is essential. The future of using mycelium for product application is promising and paves the way for a more environmentally conscious future.

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