INVESTIGATION OF THE IMPACTS OF HYBRID SUBSTRATES AND COMPACTION ON THE PROPERTIES OF MYCELIUM-BASED MATERIALS

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Abstract

Expanded polystyrene (EPS) is a type of plastic packaging widely used by the packaging industry for over 50 years. However, EPS has posed a threat to our environment, including depletion of natural resources, greenhouse gas emissions, and difficulty in degrading in the natural environment. Since 2016, many countries have passed legislation prohibiting the use of EPS worldwide. Therefore, academia and the packaging industries actively seek alternatives.

Mycelium-based materials (MBMs) have gradually gained attraction due to their sustainability and foam-like characteristics. However, concerns about the physical and mechanical properties of MBMs and the availability and cost of substrates have made both the packaging industry and customers hesitant to adopt them. This study aimed to address these concerns by upcycling waste paper and blending it with hemp hurds to reduce costs and improve certain properties. The compaction method was also explored in the study to enhance the MBMs' properties.

In the study, lower-value waste paper, a more readily available and cost-effective resource, was blended with hemp hurds to create MBMs. The physical and mechanical characteristics of the resulting samples were assessed and compared to those made of pure hemp substrates. The results showed that samples based on hybrid substrates had higher density, appeared to have better water resistance at 60% but worse at 80% RH than pure hemp hurds-based samples, but had higher values of compressive strength and Young's modulus than those made of pure hemp hurds.

In addition, the compaction method before the cultivation was used for seven protocols, and its effects on the properties of final products were assessed, with compacted and uncompacted samples for each protocol. Dry density, water absorption, and compressive properties were compared between samples with compaction and those without compaction. Compacted samples showed a 9.57-34.29% increase in dry density, a 28.57-129.63% increase in compressive strength at 10% strain, and a 37.32-139.42% increase at 35% strain, and a 27.66-142.35% increase in compressive Young's modulus. The impact of compaction on the water absorption of samples varied depending on the samples' recipes in this study.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

GL	*Ganoderma lucidum*
FF	*Fomes fomentarius*
TP	*Trametes pubescens*
HH	Hemp hurds
RH	Rice husks
MBMs	Mycelium-based materials
EPS	Expanded polystyrene

Chapter 1 Introduction

Over the past few decades, plastic materials have gradually filled our daily lives, from clothes to building materials, because they are cheaper and easier to produce in large quantities. This brings convenience to our lives but has significant impacts on our environment. The manufacture and use of synthetic plastic depletes non-renewable natural resources, such as fossil fuels and water, and contributes significantly to global warming. For example, large amounts of hydrofluorocarbons (HFCs) are released during the manufacturing process of expanded polystyrene (EPS), thousands of times more potent than carbon dioxide as a greenhouse gas (EPE, 2019). Moreover, plastic materials are not readily biodegradable, and it may take thousands of years to decompose in the natural environment, filling up our landfills and causing long-term pollution.

Packaging accounts for more than 40% of plastic production, and the amount of packaging generated is still growing astonishingly due to the increase in online shopping. Polystyrene foam has been widely used as protective packaging, but its use is now being phased out worldwide due to its negative environmental impacts. Since 2016, many countries have passed legislation to ban it. 15 countries, 12 U.S. states, and dozens of U.S. cities have prohibited the sale or use of plastic foam in food service establishments (Weighart, 2024). As a result, both academia and industry are actively looking for alternative packaging materials that are more sustainable and eco-friendly.

Mycelium-based biomaterials (MBMs) have gained significant academic and commercial interest in the past decade. These materials are created by inoculating agricultural or industrial lignocellulosic wastes with fungal mycelium. Mycelium is sometimes called the “root-like structure” of mushroom-forming fungi, and agricultural wastes provide nutrients for the mycelium to grow. The generation of MBMs results from a natural biological process without greenhouse gas emissions and non-renewable natural resource depletion. Current research on MBMs is focused on fungi species, substrates, manufacturing processes, and properties of final products. Researchers have utilized 22 fungi genres for producing MBMs so far (Aiduang et al., 2022) and substrates such as wood chips, sawdust, straw, coconut powder, garden waste, and bagasse are commonly used (Alemu et al., 2022). Besides the type of strains and substrates, the production process is crucial in determining the characteristics of MBMs. Various parameters are involved in the manufacturing process, including the inoculation method, cultivation conditions, cultivation duration, moisture level of substrates, pH level of substrates, the dehydration method, and the post-

processing method. Any changes made to these parameters would result in changes to the properties of the final product. Based on previous research, scientists usually evaluate MBMs from physical, mechanical, chemical, and biological properties.

Although MBMs have gained attraction recently, concerns about MBMs' properties, as well as costs and availability of substrates, make packaging industries and customers hesitant to choose them. This research aims to reduce production costs and improve properties by introducing waste paper in the substrates and using the compaction method in manufacturing.

In North America, a large amount of paper waste is produced annually, which is rich in cellulose (de Oliveira et al., 2023; M. Liu et al., 2023), being beneficial for improving the mechanical properties of composites. In addition, the average market price of waste paper is lower than that of hemp hurds, which may reduce the costs of raw materials for producing MBMs. Chapter 3 will elaborate on the evaluation of the impacts of using hybrid substrates of waste paper and hemp hurds on the properties of MBMs.

Pre-processing and post-processing of the materials can also affect MBMs' properties. Little data was available on the impacts of compaction on MBMs' properties. A previous study based on white-rot saprotrophic fungi and blends of the biomass materials found that samples that were densely packed before cultivation demonstrated higher values in dry density, shear moduli, Young's moduli, and compressive moduli compared to those that were loosely packed (Yang et al., 2017). Chapter 4 will focus on the impacts of using compaction before cultivation on the properties of MBMs produced from different recipes, providing a more comprehensive understanding of the role of compaction in MBMs' properties.

Chapter 2 Literature Review

2.1 Introduction

Since plastic was invented at the beginning of the last century, the number of items made from it has increased. Plastic is used for various purposes, from packaging and construction to electronics and transportation, because it offers several advantages, such as being lightweight, durable, cheap, and easily molded. However, the negative impacts of plastic on the natural environment are becoming more prominent with the increasing number of plastic products. The production of plastics consumes large amounts of natural resources, including fossil fuels and water. Plastic production accounts for 6% of global oil production, and it takes 22 gallons of water to make one pound of plastic (Water Footprint Calculator, 2022). In addition, plastics contribute significantly to global warming during the manufacturing process. For example, plastics accounted for 3.4% of global greenhouse gas (GHG) emissions in 2019, with 90% of these emissions from the production process (OECD, 2022). Furthermore, plastics are not readily biodegradable and may take more than 400 years to break down in the natural environment, most of them fill up landfills and cause long-term pollution (Parker, 2019). As of the year 2015, 60% of the plastic produced ends up in landfills or the environment forever (Cox et al., 2019). Once plastics break down into microplastics, it is more difficult to recover and recycle, causing severe harm to both terrestrial and aquatic ecosystems. In Europe and North America, around 730,000 tonnes of microplastics are transferred to agricultural lands from urban sewage sludges every year, which have adverse effects on soil, crops, and livestock (Nizzetto et al., 2016). Aquatic environments are also heavily affected by plastic waste, with 6.1 million tonnes of plastic waste entering rivers, lakes, and the ocean in 2019 alone (OECD, 2022). Microplastics present in the waterway can pose a threat to aquatic animals and have the potential to harm human health through the food chain. For example, microplastics may harm fish by causing organ damage, affecting behavior and fertility (Zolotova et al., 2022). Additionally, research has revealed that microplastics can cause harm to various systems in the human body, such as the digestive, respiratory, and immune systems (Y. Lee et al., 2023).

It is important to note that a significant portion of plastic waste is attributed to plastic packaging. In Canada, plastic packaging accounted for 63.5% and 62.7% of the plastic permanently leaked into the environment in 2012 and 2019, respectively (Statistics Canada, 2023). EPS, also termed Styrofoam, a type of plastic packaging, has been widely used by the packaging industry for over

50 years. Significant amounts of hydrofluorocarbons (HFCs) are released during manufacturing, which is thousands of times more powerful than carbon dioxide as a greenhouse gas (EPE, 2019). EPS is also the main constituent of municipal solid waste and marine debris (Rajendran, 2022). As a result, many countries have passed legislation to prohibit the use of EPS worldwide since 2016. In Canada, there is an initiative to reach zero plastic by 2030. To achieve this goal, a ban on EPS sales started at the end of 2023, with a complete prohibition on manufacturing, importing, and exporting sales going into effect by the end of 2025 (Lee, 2022). It means that environmentally friendly alternative materials should be explored.

MBMs are a new type of biomaterial that has emerged in recent decades and have the potential to replace EPS. The raw materials for producing MBMs are mycelium and agricultural lignocellulosic wastes. During manufacturing, the mycelium acts like a "pipeline system" infiltrating the substrate and thereby acting as a natural glue, releasing enzymes that break down the substrate and thereby binding the waste materials to the network of hyphae that make up the individual strands of the expanding mycelium. The end product can be a bio-foam-like material suitable for packaging (as Styrofoam replacement) or construction materials.

MBMs have several significant environmental benefits compared to synthetic materials. These include 100% biodegradability, lower carbon footprint, reduced energy consumption, and upcycling wastes. Table 2.1 presents a comparison between MBMs and EPS in terms of eco-friendliness.

Table 2.1 Comparison between MBMs and EPS

	MBMs	EPS
Biodegradability in the natural environment	Completely biodegradable	High resistance to natural degradation (Palmer et al., 2022)
Lifespan in nature	Several months (Gan et al., 2022; Van Wylick et al., 2022)	More than 500 years (Davis, 2019)
Embodied carbon	Negative (Livne et al., 2022)	224 CO_2 eq m^{-3} (Crawford et al., 2019)

Embodied energy	860 MJ m^{-3} (Livne et al., 2022)	2710-3565 MJ m^{-3} (Livne et al., 2022)
Energy consumption for producing 1 kg material	4-6MJ (Enarevba & Haapala, 2023)	42.4MJ (Enarevba & Haapala, 2023)

MBMs are composed of natural materials without synthetic ingredients and are designed to biodegrade in the environment. Recent research has confirmed this fact. It took less than two months for MBMs made from Oyster mushrooms and bamboo to degrade in the soil in a tropical environment (Gan et al., 2022). The MBMs composed of *Ganoderma resinaceum* and hemp fibers lost 43% of their weight after being buried in soil with a temperature of 25 ℃ and relative humidity of 50% for 16 weeks (Van Wylick et al., 2022). The biodegradation test of MBMs using hemp and wood as substrates showed a mass loss above 70% after composting for 12 weeks in a compost pile containing grass and hardwood sawdust under 60℃ (Zimele et al., 2020). Although scientists applied different biodegradability testing methods in the research, all the results, without exception, prove that MBMs are completely biodegradable. Therefore, the amount of solid waste buried in landfills could be reduced, if EPS were replaced by MBMs.

MBMs have a lower carbon footprint, less natural resource requirement, and fewer gas emissions. The embodied energy (EE) of MBMs was estimated to be 860 MJ m^{-3}, while the EE for EPS was estimated to be 2710-3565 MJ m^{-3} (Livne et al., 2022). In another research, energy requirements for producing MBMs and Styrofoam were reported to be 4-6 MJ/kg and 42.4 MJ/kg, respectively (Enarevba & Haapala, 2023). The amount of water consumed for producing MBMs and EPS is around 144 kg/m^3 (de Bruin, 2019)and 2088-8700kg/m^3 (British Plastics Federation, 2024; I. Boustead, 2005), respectively. As for the greenhouse gas emissions, a comprehensive life cycle assessment reported that MBMs have a negative embodied carbon (EC) value of -39.5 kg CO_2 eq m^{-3} and act as a CO_2 sink (Livne et al., 2022), while the EC value of EPS is 224 CO_2 eq m^{-3} (Crawford et al., 2019).

Furthermore, MBMs can upcycle agricultural by-products that have lower value rather than utilize non-renewable natural resources as raw materials, tapping the economic potential of local agricultural wastes. A research team at the University of British Columbia used the wood waste

that is left behind after tree harvest to develop MBMs, which help the First Nation create the forest bioeconomy (Bosshart, 2022).

Besides these advantages, MBMs possess competitive physical and mechanical properties, including low density, water resistance, sound absorption, great compressive strength, and compression modulus. These properties make them a promising choice for various purposes in different industries under the goal of net-zero emission.

2.2 Research Status of MBMs

The initial research on MBMs was documented in 1966, and for several decades, the number of articles published each year on the topic remained relatively stable (Alaneme et al., 2023). However, in 2008, a significant upward trend in the number of publications can be seen despite some fluctuations (Alaneme et al., 2023). After 2018, the number of publications skyrocketed (Alaneme et al., 2023). That means MBMs have gained significant academic attention in recent years due to the growing concern about global warming worldwide. Scientists focus on improving the properties of MBMs by experimenting with different strains, substrate types, and manufacturing methods. Although there is no standard and uniform manufacturing protocol for MBMs, according to current literature, most strategies involve five components: fungal strain selection, substrate preparation, inoculation strategies, cultivation conditions, and dehydration process.

2.2.1 Fungal Strain Selection

Fungi are widely distributed on Earth, and less than 2% of the estimated 4-5 million species have been described so far (Gautam et al., 2022). Fungi's methods of vegetative growth and nutrient intake make them distinguished from other living organisms. They grow by extending hyphae and digesting organic matter externally before absorbing nutrients into the mycelium.

There are around 70 fungal species being utilized to produce MBMs between 2012 and 2022, including white rot, brown rot, and soft rot. White rot accounted for 85% of these species, while brown rot and soft rot contributed to the remaining 15% split almost evenly (Sydor et al., 2022). White-rot fungi are the most effective for degrading lignocellulose materials because they can produce ligninolytic extracellular oxidative enzymes (Abdel-Hamid et al., 2013). Some species of white-rot fungi break down lignin more efficiently than hemicellulose and cellulose, leaving

enriched cellulose, while others degrade all cell wall components simultaneously, forming radial cavities (Wong, 2009). Unlike white-rot fungi, brown-rot fungi and soft-rot fungi both digest cellulose and hemicellulose of the substrates rather than lignin. More cellulose contained in the residue can enhance the physical properties of MBMs, which might be why white rot fungi are commonly used in their production.

Scientists consider the hyphal extension rate an essential factor when choosing fungal species for MBMs, as it can influence the production rate. Previous research showed that the hyphal extension rate is associated with different types of hyphae - monomitic, dimitic, and trimitic. White rot and brown rot belong to Basidiomycetes, in which monomitic species contain generative hyphae, dimitic species contain generative-binding hyphae or generative-skeletal hyphae, and trimitic species contain generative-binding-skeletal hyphae (Webster & Weber, 2007). Although there are variations in growth rate within and between different types of hyphae, it was observed that monomitic species that only contained generative hyphae had a slower extension compared to trimitic species (Jones et al., 2018). The average extension rate of monomitic species over 7 days was 41mm, while that of trimitic species was 63mm (Jones et al., 2018). It was also observed that dimitic species with generative-skeletal hyphae demonstrated a higher extension rate than those with generative-binding hyphae (Jones et al., 2018). However, there is limited research on the hyphal extension rate of Ascomycota, to which soft rot fungi are assigned. One study that compared the mycelium extension rate of 49 different fungal species showed that the growth rate of *Ascomycota* on PDA was higher than that of *Basidiomycota* (Hannula et al., 2023).

The impact of different species on the characteristics of MBMs is another factor researchers are considering. Studies have shown that the fungal species selected could affect MBMs' density. For instance, MBMs fed with *Agaricus bisporus* and rapeseed cake had a higher density than that of MBMs produced from *Ganoderma lucidum* with the same substrate (Tacer-Caba et al., 2020). As for the relationship between the water absorption of MBMs and the chemical compositions of the strain's cell wall, there is no consistent conclusion. Some studies have discovered that MBMs fed with *P. ostreatus* and cellulose-potato dextrose broth had high humidity sensitivity under 100% relative humidity due to a relatively lower content of chitin in the *P. ostreatus* cell wall (Haneef et al., 2017). However, for MBMs produced from strains of *Trichoderma asperellum*, *Lentinula edodes*, *Ganoderma Lucidum*, *Pleurotus ostreatus sajor caju* and the substrates of rapeseed and

oat husk, the obvious relationship between water uptake and the strength of chitin signals in the Fourier transform infrared (FT-IR) was not discovered (Tacer-Caba et al., 2020). In addition, previous research has indicated that the cell wall composition of strains affects MBMs' mechanical performance. *P. ostreatus*-based MBMs have higher stiffness (E: 28MPa) than *G. lucidum*-based ones (E: 12Mpa) due to the higher polysaccharides' content in the *P. ostreatus*-based MBMs, and the larger elongation in *G. lucidum*-based MBMs (ε: 33%) compared to *P. ostreatus*-based ones (ε: 9%) is consistent with a large amount of proteic and lipid constituents in *G. lucidum*-based materials (Haneef et al., 2017). Overall, the cell wall composition of strains plays an important role in the MBMs' performance, especially the presence of chitin, proteins, lipids, and polysaccharides. Chitin helps in aggregating particles of substrates, providing mechanical strength and reducing crack formation during compression (Teixeira et al., 2018; Yang et al., 2017). Proteins and lipids in the cell wall might act as plasticizers, while polysaccharides can offer stiffness to MBMs (Haneef et al., 2017).

In addition, it is essential to select fungi capable of breaking down various lignocellulose materials, making it possible to utilize a wide range of wastes, which in turn may enhance the cost efficiency of the production process (Sydor et al., 2022).

2.2.2 Substrates Selection and Preparation

(1) Substrates Selection

Agricultural by-products, industrial wastes, and post-consumer wastes are the primary sources of substrates for producing MBMs (Sydor et al., 2022). Table 2.2 shows some substrates utilized in the previous studies.

Table 2.2 Substrates used to produce MBMs and their nutritional contents

Substrate type	Lignin (%)	Cellulose (%)	Hemicellulose (%)	Reference
Hemp fibers	6	58.7	14.2	(Le Troedec et al., 2008)
Hemp hurds	21.03	44.5	32.78	(Stevulova et al., 2014)
Pinewood	24	42	23	(Nakarmi et al., 2020)

Oakwood	25.71	41	26.35	(Le Floch et al., 2015)
Cotton fibers	1	88-96	-	(Maundrill et al., 2023)
Cotton seed hulls	17-19	30-32	~20	(Cheng & Biswas, 2011)
Wheat bran	5	11	11-26 (other constituents included)	(Glaser et al., 2023)
Flax	~24	~53	~24	(Ross & Mazza, 2010)
Jute	15.75	62.35	19.51	(Hossen et al., 2020)
Rice husk	20-25	35-40	15-20	(Gao et al., 2018)
Beech sawdust	15-25	38-50	23-32	(L. Liu et al., 2023)
Rapeseed straw	17.72	49.52	12.69	(Ji et al., 2014)
Rice straw	5-24	32-47	19-27	(Ngo et al., 2022)
Wheat straw	20-22	32-41	22-28	(Rodríguez-Sanz et al., 2022)
Corn straw	10	30-40	25-30	(Song et al., 2020)
Bagasse	~25	~50	~25	(Zhou et al., 2016)
Coir pitch	31	28	9.5	(Das Rabindranath, 2012)
Waste Paper	2.0-34.5	30.5-79.2	3.5-20.3	(de Oliveira et al., 2023)

Nutrition content is the most important factor that should be considered when choosing substrates (Osaymen Precious, 2019). All the substrates listed in Table 2.2 are lignocellulose materials, which are composed of 1-31% lignin, 11-96% cellulose, and 11-32.78% hemicellulose. It is important to provide ample nitrogen and carbohydrates during production to promote rapid mycelium growth. For example, *P. ostreatus* mycelium fibers grow faster on wheat bran ($1.5 \pm 0.2 cm/day$) and sugarcane ($1.5 \pm 0.2 cm/day$) than on sawdust ($0.66 \pm 0.1 cm/day$) because the former substrates are rich in nutrients that promote growth rate (Joshi et al., 2020). However, substrates

like wheat bran, which are rich in nutrients, might increase the risk of contamination by providing nutrients to other microorganisms (Joshi et al., 2020). Nutritional content in the substrates can also affect the final product's properties. The tensile strength of MBMs made from beech sawdust is $0.05MPa$, which is lower than those made from red oak sawdust which is $0.18MPa$ because the tensile properties are significantly influenced by the nutrient content of the substrates (Jones et al., 2020). MBMs made with *G. lucidum* or *P. ostreatus* and cellulose substrates presented higher Young's modulus and lower elongation than those fed with the same strains and dextrose-containing substrates because mycelium needs to synthesize more chitin to penetrate substrates when being fed with pure cellulose, which is more difficult to hydrolyze (Haneef et al., 2017). As a result, the increasing synthesis of chitin leads to a higher ratio of chitin to polysaccharides in MBMs, which would improve their mechanical strength and reduce ductile behavior. In addition, some researchers used supplements to improve the nutritional content of substrates. For instance, 10% wheat bran added to substrates for mycelium cultivation can help improve the mycelium growth rate and compressive strength and lower the dry density because wheat bran offers mycelium more nitrogen, leading to better and faster mycelium growth (Ghazvinian et al., 2020). Supplements used in previous studies include wheat straw, wheat bran, cotton bran, cotton seed hull, starch, cellulose fiber, hay, glycerol, molasses, and potato dextrose broth (Alemu et al., 2022; Haneef et al., 2017). However, it is worth noting that different fungal species have different nutritional preferences. *P. ostreatus* utilized hemicellulose as the primary energy and carbon source and left cellulose almost intact (Chi et al., 2007), while *Ganoderma lucidum* preferred substrates with a high amount of cellulose and lignin, a low amount of nitrogen, and a high cellulose: lignin ratio (Atila, 2020). Therefore, the nutrients of substrates considered when selecting substrates should be related to fungal species' food preferences.

When selecting substrates, their physical and mechanical properties are usually considered. However, there are limited and inconsistent results regarding the impact of these properties on the mechanical and physical properties of MBMs. For the density, MBMs containing agricultural by-product substrates have lower densities ($60-130\ kg/m^3$) than those produced from forestry by-product substrates ($87-300\ kg/m^3$) (Jones et al., 2020). For the compressive properties, one study has shown that the physical form of agricultural by-product fibers can exert an influence (Elsacker et al., 2019). For instance, using chopped hemp ($<5mm$) and chopped flax ($<5mm$) leads to higher compressive stiffness values of 0.77MPa and 1.18MPa, respectively, compared to

loose hemp and loose flax, which have compressive stiffness values of 0.51MPa and 0.28MPa, respectively (Elsacker et al., 2019). In addition, researchers also found that particulate substrates, such as sawdust, provide higher compressive properties to the MBMs than fibrous substrates, such as straw (Jones et al., 2020), and MBMs' compressive properties are not significantly affected by the particle size of the substrates used (Islam et al., 2018). For the flexural strength of MBMs, it is not influenced obviously by the particle geometry of substrates (Jones et al., 2020). For the tensile properties, Jones et al. (2020) mentioned that it is not necessarily linked to the mechanical properties of the substrates based on the research conducted by Appels et al. (2018) and Travaglini et al. (2013), which indicated that MBMs fed with red oak sawdust had a much higher tensile strength (0.18MPa) than those fed with beech sawdust (0.05MPa) (Appels et al., 2019; Travaglini & Noble, 2013) though red oak has a similar tensile strength to beech wood (5.5 MPa vs 5-7 MPa) (Appels et al., 2019; Travaglini & Noble, 2013). Nevertheless, Appels et al. (2018) used Trametes multicolor as the strain in the research, and Travaglini et al. (2013) used *Ganoderma lucidum* as the strain for producing MBMs (Appels et al., 2019; Travaglini & Noble, 2013). Therefore, it isn't easy to draw a conclusion about the impact of the tensile strength of substrates on the tensile strength of MBMs without considering the fungal species used. Overall, scientists found that the mechanical properties of MBMs depend on the mycelium growth situation more than the physical and mechanical properties of substrates themselves, irrespective of loading conditions (Jones et al., 2020). Nevertheless, mycelium growth is significantly impacted by fungi species and the nutrient profile of substrates, as mentioned above.

Finally, the availability of substrates is vital for producing MBMs on a large scale and reducing the transportation costs of raw materials. Therefore, scientists always try to use locally available wastes to produce MBMs. For example, research on the effect of growth factors conducted in Malaysia utilized rice husk and sugarcane bagasse as substrates (Nashiruddin et al., 2022), whereas studies about producing humidity-resistant MBMs conducted in Finland used oat husk and rapeseed cake as substrates (Tacer-Caba et al., 2020). Beech sawdust was chosen as the initial substrate in the research because of its local availability (Vašatko et al., 2022). A study in Australia chose rice hulls as substrates because they are readily available in large quantities, with 167 million tonnes produced worldwide annually (Jones et al., 2018).

(2) Substrates Preparation

Before inoculating substrates with suitable fungal biomass, substrates need to be hydrated and sterilized.

There are two ways to humidify substrates. Soaking the substrates in water is the usual method, and soaking duration depends on the water absorption capacity of the substrates (Jones et al., 2020). Beech sawdust was soaked in distilled water for 24 hours (Vašatko et al., 2022), while rice hull and wheat grain were soaked in water for 48 hours (Jones et al., 2018). The chopped straw, banana leaf midribs, and Mehegoni leaves were soaked in water for 12 hours in the research about the performance of Oyster mushrooms on different substrates (Hasan et al., 2010). If the substrate is not humidified before sterilizing, a certain amount of sterile water can be added to the substrate before inoculation. For instance, in a study about the properties of MBMs made from different substrates, 20% wt (weight percentage) of fibers, 70% wt of sterile demineralized H_2O and 10% wt of mycelium spawn were mixed together before putting in the moulds for incubation (Elsacker et al., 2019).

Humidified substrates must be sterilized to eliminate microorganisms that already exist on the substrates. Autoclaving is the major sterilization method to remove potential microbial contamination. Substrates can be autoclaved at 121℃for 15 to 60 minutes, depending on the amount and density of the substrates (Arifin & Yusuf, 2013; Attias et al., 2017; Bruscato et al., 2019; Elsacker et al., 2019; Elsacker et al., 2022; Haneef et al., 2017; Jones et al., 2018; Jones et al., 2019; Matos et al., 2019; Nashiruddin et al., 2022). Sometimes the autoclaved temperatures are lower than the usual 121℃, such as 115℃ and 100℃ (Attias et al., 2019; Xing et al., 2018). Pasteurization is another sterilization method used in the research (Nguyen et al., 2022; Ridzqo et al., 2020; Yang et al., 2017). For instance, materials were pasteurized at 115℃ for 28 minutes at an Ecovative's pilot manufacturing plant (Holt et al., 2012). Hydrogen peroxide is also used to sterilize substrates, with a concentration of 0.3% or 10% (Jiang et al., 2017; Lelivelt et al., 2015). In addition, some other sterilization methods have been used in previous research. Nava et al. (2016) put substrates in the oven at 85℃ for 120 minutes to disinfect. Irbe et al. (2021) put the decortication fibers in 4% NaOH solution at 165℃ for 75 minutes before washing them with tap water to neutralize them. Răut et al. (2021) sterilized substrates with 70% ethanol before rinsing them with sterile water and then put them under UV radiation for 10 minutes. Sun et al. (2022) used steam to sterilize substrates at 121℃ for 60 minutes. It is worth noting that substrates

sterilized by autoclaving at 15 lbs. pressure for 20 minutes took less time for spawn run when compared to those that were sterilized with hot water (30 minutes), formaldehyde solution (50 mL/L water), or bavistin (2 g/L water) (Atila, 2016; Kalita, 2015).

2.2.3 Inoculation Strategies

The substrates can be inoculated with liquid inoculum, agar inoculum, or grain spawn. Liquid inoculum could provide a more consistent and even distribution of spores throughout the substrates compared to grain spawn (Holt et al., 2012). Fungi grown in liquid cultures can be homogenized to generate smaller fragments, and some fungi can sporulate in liquid media. However, it is difficult for the mycelium to grow and spread without enough nutrients in the solution. Lack of nutrients may lead to slow growth or failure of mycelium growth. There is limited paper about liquid inoculum and its formula. A previous study mentioned a liquid inoculum formula, which contains a filtered liquid (20 wt% cotton bran, 10 wt% corn flour, and 1000 ml water), 1 wt% KH2PO4, 1 wt% K2HPO4, 0.5 wt% MgSO4, 20 wt% glucose, and *Ganoderma lucidum* (Liu et al., 2020). Agar inoculum usually uses agar plates, such as potato-dextrose agar plates or malt extract peptone agar plates, as media to grow fungal species until mycelium covers the whole surface of the agar plate. The fully colonized plates can be cut into small pieces and inoculated directly in the substrates. In addition, various types of grains, such as wheat, rye, brown rice, white rice, wild bird seed, and popcorn (Shields, 2020), can be sterilized and inoculated with suitable fungi, and after a period of incubation, the grains have been colonized by the fungi mycelium. Using grain spawn as a source of inoculum can mitigate the initial growth difficulties of the mycelium because the grain can provide a rich source of nutrients for fungal development. Inoculation of substrates with grain spawn can increase the success of mycelium colonization of lower-nutrient substrates.

It is important to consider the amount of inoculum as it can affect mycelium growth efficiency and production cost. Previous research recommended using 10-32 wt% of any form of inoculum (Jones et al., 2020) or a <20% volume/volume of liquid inoculum (Schaak & Lucht, 2016) for the substrates.

Different inoculation methods may impact the properties of MBMs. Research using both liquid inoculation and grain-based inoculation methods discovered that MBMs with liquid inoculation

have higher flexural Young's modulus and compressive strength, while MBMs with grain inoculation have higher density (Holt et al., 2012).

2.2.4 Cultivation Conditions

The inoculated substrate essentially forms a matrix composed of a fungal network (mycelium) that colonizes the substrate particles. This matrix is usually placed into a mold that provides the desired shape and dimensions of the MBMs, and the mold is usually put in a dark or low-light environment that helps mycelium grow. The molds supporting the development of MBMs must support airflow for oxygen to be available for the fungus and prevent moisture loss. Incubation of the molds needs attention that maximizes growth rates for the mycelium. Previous studies have shown that temperature, moisture levels in the substrates, relative humidity (RH), and cultivation duration are important factors for the cultivation of mycelium and the efficient generation of MBMs.

Incubation temperature depends on the fungi species utilized for generating the mycelium, and in most studies, it ranges from 20℃ to 30 ℃ (Bhardwaj et al., 2021; Läkk et al., 2018; Lee & Choi, 2021; Răut et al., 2021; Stelzer et al., 2021; Tacer-Caba et al., 2020; Travaglini et al., 2016; Vidholdová et al., 2019). However, some studies set lower or higher incubation temperatures for mycelium growth. For instance, research focused on enhancing termite resistance of MBMs incubated materials at 2 ℃ for 5 days (Bajwa et al., 2017). Another study aimed to evaluate the acoustic properties of MBMs, while yet another study used bamboo as substrates incubated inoculated materials at 30-35 ℃ (Pelletier et al., 2019; Ridzqo et al., 2020).

Few studies specify the exact moisture level of the substrate. Sufficient moisture content in the substrate facilitates the growth of mycelium. However, some studies discovered that the mycelium growth rate is slower when the moisture level of the substrate reaches 60% or higher, and excessive moisture may lead to anoxic conditions, which can decrease the enzymatic activities of the fungal and encourage contamination by molds or bacteria (Nashiruddin et al., 2022).

The ideal RH for mycelium growth is typically between 80% and 90% (Cerrato, 2018). Mycelium will not develop properly and may die if RH is too low. Mycelium will grow faster in a high RH environment, but a high RH value can make the mycelium-generated material more susceptible to mold confirmation and growth. However, there is a wide range of RH values in previous studies from 50% to 100% (Antinori et al., 2020; Attias et al., 2019; Gou et al., 2021; Jiang et al., 2016;

Jose et al., 2021; Lee & Choi, 2021; Lelivelt et al., 2015; Liu et al., 2019, 2020; Özdemir et al., 2022; Soh et al., 2020; Sun et al., 2022; Jones et al., 2018).

Cultivation duration also affects MBMs' properties and potential application. It can range from 5 to 56 days (Attias et al., 2017; Jiang et al., 2016; Matos et al., 2019; Shao et al., 2016; Xing et al., 2018; Ghazvinian & Gürsoy, 2022). The increased period of mycelium growth can improve thermal stability and compressive strength but lead to a negative impact on the elastic and shear moduli (Yang et al., 2017).

2.2.5 Dehydration Process

After the mycelium/substrate combinations have been developed and taken out of the mold, there are several ways to dehydrate MBMs, including oven, microwave heating, thermal pressing, drying machine, IR lamp, and air drying at room temperature (Arifin & Yusuf, 2013; Holt et al., 2012; Jiang et al., 2013; Lelivelt et al., 2015; Yang et al., 2017). The temperature required for dehydration varies between 50℃ and 250℃, and the duration of dehydration ranges from 1 to 72 hours, depending on the chosen drying method. When using the oven, materials were often dried at 50℃-125℃ for 2-48 hours (Ahmadi, 2016; Bajwa et al., 2017; Jiang et al., 2016; Lelivelt et al., 2015; Sun et al., 2022). When using hot pressing, materials were usually pressed at 160℃-250℃ for 6-20 minutes (Jiang et al., 2016, 2017; Liu et al., 2020; Sun et al., 2022).

Changes in sample weight after drying and post-dry moisture content of the MBMs are two other factors to consider when to stop dehydration. One study stopped drying materials until the weight was reduced to less or equal to the original weight of the mycelium-generated sample (Moser et al., 2017). A video from Ecovative company shows that dehydration can be stopped when baking to 35% of the original weight or when the MBMs have a moisture content of 5-12% (Ecovative Design, 2018).

2.2.6 Pre-pressing and Post-pressing

Some studies tried to improve MBMs' properties by pre- or post-pressing. A study aimed at researching MBMs' properties under different protocols and packaging methods shows that samples that were densely packed before cultivation demonstrated higher values in dry density, shear moduli, Young's moduli, and compressive moduli compared to those that were loosely packed (Yang et al., 2017). In addition, post-pressing can impact the tensile strength, elastic

modulus, flexural strength, and flexural modulus of MBMs (Appels et al., 2019). For heat pressing samples made from *Pleurotus ostreatus* and rapeseed straw, the tensile strength (0.24±0.03 Mpa), elastic modulus (97±9.0Mpa), flexural strength (0.87±0.14 Mpa), and flexural modulus (72±6.6 Mpa) are significantly higher than those with cold pressing (tensile strength of 0.03±0.00 Mpa, elastic modulus of 9±1.2Mpa, flexural strength of 0.21±0.01Mpa, and flexural modulus of 15±1.1 Mpa) and no pressing at all (tensile strength of 0.01±0.00 Mpa, elastic modulus of 2± 0.3MPa, flexural strength of 0.06±0.01Mpa, and flexural modulus of 1±0.4 Mpa) (Appels et al., 2019). Recent research also discovered that immersing samples produced from *Ganoderma lucidum* and cotton stalk in distilled water until they uptake 30 wt% of water prior to heat pressing can enhance flexural and internal bonding properties (Liu et al., 2020).

2.2.7 Properties of MBMs

Previous research often evaluates the physical and mechanical properties of MBMs.

Density and water absorption are important physical traits. The density of MBMs could affect transportation cost and water absorption decides the application scenario of MBMs. Previous research demonstrated that MBMs' density varies between 25-954 kg/m^3 (Angelova et al., 2021; Attias et al., 2019; Bruscato et al., 2019; Chan et al., 2021; Dias et al., 2021; Ghazvinian et al., 2020; Gou et al., 2021; Joshi et al., 2020; Kuribayashi et al., 2022; Nashiruddin et al., 2022; Schritt et al., 2021; Teixeira et al., 2018), and some results showed that MBMs could possess low density similar to EPS. Samples fed with oat husks and various fungal species, such as *Ganoderma lucidum*, *Agaricus bisporus*, and *Pleurotus ostreatus*, have a density of 25.0 kg/m^3, 36.0 kg/m^3, 38 kg/m^3, respectively (Tacer-Caba et al., 2020). These densities are comparable to or within the range of synthetic foams' densities, which are between 11-120 kg/m^3 (Jones et al., 2020). The water absorption performance of MBMs ranges from 24.45% to 508% (Angelova et al., 2021; Appels et al., 2019; Dias et al., 2021; Elsacker et al., 2019; Gou et al., 2021; Joshi et al., 2020; Kuribayashi et al., 2022; Lee & Choi, 2021), which is significantly higher than that of EPS that is 0.3%-4.0% (Nava et al., 2016)It is worth noting that the testing methods for water absorption in previous research varied, and the International Organization for Standardization (ISO) and the American Society for Testing and Materials (ASTM) were the main methods used.

Mechanical properties usually comprise compressive strength, tensile strength, and flexural strength. These properties are crucial parameters to test the potential of MBMs replacing EPS because they represent the ability of the material to withstand loads, the extent of stretch before breaking, and the resistance to deformation. The compressive strength, tensile strength, and flexural strength of EPS are 0.069-0.4MPa, 0.014-0.023 MPa, and 0.07-0.69MPa, respectively (Nava et al., 2016). On the other hand, MBMs presented competitive mechanical characteristics with compressive strength ranging from 0.03 to 4.44 MPa, tensile strength ranging from 0.01 to 1.55 MPa, and flexural strength ranging from 0.05 to 4.40 MPa (Aiduang et al., 2022)This shows that MBMs have the potential to substitute EPS in terms of mechanical properties.

2.3 Application Status of MBMs as Packaging Materials

Research and industry efforts have shown the potential of using MBMs as packaging materials. The cotton-based MBMs demonstrate similar physical and mechanical properties to extruded polystyrene foam (Holt et al., 2012). MBMs made from *Pleurotus Ostreatus* oyster mushroom and mixture substrates of saw-dust and coir-pith showed competitive mechanical properties compared to EPS, making it a potential alternative to EPS in packaging applications (Sivaprasad et al., 2021). Mycelium composites produced by *Trametes versicolor*, birch or poplar sawdust, and other supplement nutrients have promising potential to substitute lightweight plastics in transportation packaging (Zhang et al., 2023). In addition, MBMs are currently on the market in Europe, Indonesia, and the United States, and they are mainly applied for non-structural building applications because of their foam-like properties, including packaging materials. Ecovative, a mycelium technology company founded in 2007 in the USA, uses hemp hurds and mycelium to produce mushroom packaging and has successfully collaborated with several companies. Magical Mushroom Company, based in the UK, produces protective mushroom packaging at any scale and has already produced more than 2.5 million pieces of mushroom packaging since 2020 (Magical Mushroom Company, 2023). S.lab, a Ukrainian startup, has completed a pilot for cosmetics maker L' Oreal, using its mushroom-based packaging to cushion shampoo and conditioner packs (Boztas, 2023). Grown Bio, a Netherlands biotechnology company, focuses on producing mycelium-based protective packaging, including industrial packaging, luxury packaging, insulating packaging, and influencer packaging. Moreover, some significant companies have used mycelium-based packaging for their products. Dell began using mushroom packaging as cushions to protect some heavier shipments in North America in 2011 (Dell, n.d.). IKEA announced its use of mushroom-

based packaging in 2019, reducing the use of polystyrene-based materials (Emenac Packaging Canada, 2021).

Although MBMs have many advantages as eco-friendly biomaterials, several challenges may limit their wide use in the packaging industry. Shelf life is an essential issue that packaging industries are concerned about. Since mycelium-based materials are entirely compostable, they always have a relatively short shelf life. For example, samples made from *Pleurotus Ostreatus* oyster mushroom and a mixture of substrates of saw-dust and coir-pith could only remain physically intact for 67 days under indoor conditions (Sivaprasad et al., 2021). This kind of mushroom packaging may not be suitable for cosmetic products, which usually have a shelf life of 3 years in Canada (Nguyen, 2019). The feasibility of using MBMs as food packaging is worth exploring, though there are few studies in this area. Unlike other products, food may not require packaging with a long lifespan as other products. MBMs are a good option as they are home-compostable, which can reduce the amount of plastic food packaging that ends up in landfills. However, food packaging should meet local safety standards to ensure that no harmful chemicals are transferred to the food. Even if the raw materials are non-toxic, the final products of MBMs need to be tested for non-toxicity in accordance with food packaging requirements. Another challenge is the poor water resistance of MBMs compared to petroleum-based materials, limiting their use in wet environments or contact with liquids. Furthermore, the accessibility of raw materials and the potential to tailor the end product by incorporating features like coloring and printing labels or brand names on the MBMs are crucial to the industry. These requirements could be subject to further research to enhance the marketability of mycelium-based packaging materials.

2.4 Conclusion

MBMs are environmentally friendly throughout their life cycle. They are produced from natural, low-cost materials, and the manufacturing process is based on a natural biological process of fungal growth without greenhouse gas emissions and non-renewable resource depletion. MBMs can be naturally biodegraded in the environment at the end of use instead of ending up in landfills. Current academic research focuses on the manufacturing process, screening for mycelium-generating species and suitable substrates, and improving the physical and mechanical properties of MBMs. Meanwhile, MBMs have been used as packaging materials in the industry, but there is

still a long way from being widely used as packaging in our lives due to some challenging issues that need to be resolved, such as water absorption, shelf life, and printability.

Chapter 3 Investigation of using hybrid substrates of waste paper and hemp hurds to produce mycelium-based materials

Abstract

Academia and industries have shown a growing interest in mycelium-based materials (MBMs) for eco-friendly packaging due to their sustainability and foam-like properties. Nevertheless, for scaled production, the availability and cost of substrates are issues that cannot be ignored. Agricultural or industrial lignocellulosic byproducts are commonly utilized as substrates for producing mycelium-based materials. However, certain byproducts like hemp hurds have been repurposed for alternative uses, resulting in the potential challenges of limited availability and increased costs. This study blended lower-value waste paper, being more readily available and cost-effective, with hemp hurds to create mycelium-based materials. The physical and mechanical characteristics of the resulting samples were assessed and compared to those fed with pure hemp substrates. The results showed that samples based on hybrid substrates had higher density, appeared to have better water resistance at 60% but worse at 80% RH than pure hemp hurds-based samples, and performed higher compressive properties than those made of pure hemp hurds.

Keywords: fungal mycelium; waste paper; hybrid substrates; mycelium-based biomaterials

3.1 Introduction

The negative impacts of plastics on the natural environment are becoming increasingly prominent, including natural resource depletion, greenhouse gas emissions, and non-biodegradability in the natural environment. A significant portion of plastic waste is attributed to plastic packaging. Expanded polystyrene (EPS) is a type of plastic that has been a prevalent choice in the packaging industry for over 50 years. Significant amounts of hydrofluorocarbons (HFCs) are released during manufacturing EPS, which is thousands of times more potent than carbon dioxide as a greenhouse gas (EPE, 2019). EPS is also the main constituent of municipal solid waste and marine debris (Rajendran, 2022). Since 2016, many countries have passed legislation to ban EPS. As a result, academia and industry are actively seeking alternative packaging materials that are more sustainable and eco-friendly.

Mycelium-based materials (MBMs) are a new type of biomaterial that has emerged in recent decades and can potentially replace expanded polystyrene (EPS). During manufacturing, the

mycelium acts like a “pipeline system” infiltrating the substrate and thereby acting as a natural glue, releasing enzymes that break down the substrate and thereby binding the waste materials to the network of hyphae that make up the individual strands of the expanding mycelium. The end product can be a bio-foam-like material suitable for packaging materials. Compared to plastic materials, MBMs are completely biodegradable (Gan et al., 2022; Van Wylick et al., 2022; Zimele et al., 2020) and have a lower carbon footprint with little gas emissions and natural resource requirements. (Crawford et al., 2019; Enarevba & Haapala, 2023; Livne et al., 2022). Furthermore, previous research has shown the potential of using MBMs as packaging materials regarding their physical and mechanical properties (Holt et al., 2012; Sivaprasad et al., 2021; Zhang et al., 2023).

The substrate plays an essential role in both academic research and commercial application of MBMs, influencing the properties of final products and the manufacturing cost. Substrates containing lignin, cellulose, and hemicellulose are frequently utilized, as they are favored by white rot fungi, commonly used for producing MBMs for colonization. Agricultural by-products, industrial wastes, and post-consumer wastes are the primary sources of substrates for producing MBMs (Sydor et al., 2022). Hemp hurds are among the most popular substrates for MBMs’ production due to their nutritional content and mechanical properties. However, animal bedding, construction materials, and garden mulch utilize a substantial portion of hemp hurds’ total applications (Carus & Sarmento, 2016), potentially impacting the availability and pricing of raw materials for MBMs’ production. Exploring abundant lower-value materials as substrates offers a promising approach to mitigate this concern.

There is a large amount of paper waste available annually in North America. In 2019, 110 million tons of paper and cardboard waste were managed in the USA, with 56% of the amount ending up in landfills (Milbrandt et al., 2024). Statistics Canada reported that Canada exported 255,327 tonnes of waste paper to India in 2022 (Dayal, 2023). Furthermore, the average market price of waste paper ranged from $35 to $135 per ton between 2019 and 2021, which is lower than the price of hemp hurds, approximately $200 per ton in 2021 (Milbrandt et al., 2024; New Leaf Data Services, 2021). In addition, waste paper is rich in cellulose (de Oliveira et al., 2023; Liu et al., 2023), which is beneficial for improving the mechanical properties of composites. Some research has demonstrated that incorporating paper into different polymers can improve the physical and

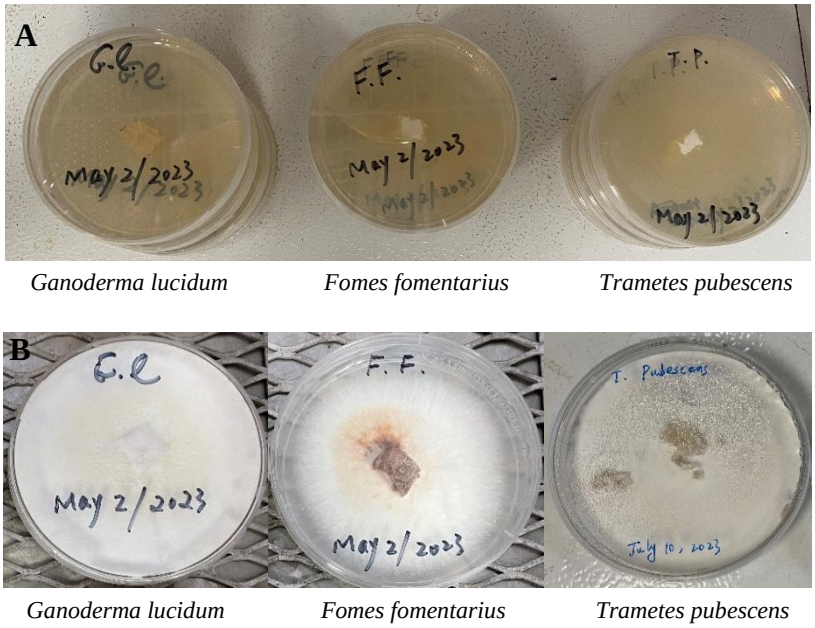
mechanical characteristics, including tensile strength and stiffness of the composites (de Oliveira et al., 2023).

Therefore, this study explores the viability of upcycling waste paper as filler within hemp hurds substrates for producing MBMs. Two different paper ratios were employed to evaluate the effects of varying proportions of waste paper. Three different mycelium strains were applied in the research to evaluate the compatibility of these hybrid substrates. Each strain underwent three protocols: one utilizing pure hemp hurds as a control, while the other two involved hybrid substrates. The physical and mechanical properties were assessed for the final samples to figure out the impacts of hybrid substrates.

3.2 Materials and Methods

3.2.1 Initial Fungal Cultures

The fungal species *Ganoderma lucidum*, *Fomes fomentarius*, and *Trametes pubescens* were obtained from the Department of Microbiology at the University of Manitoba. All strains were inoculated on the malt extract agar (MEA) medium under a sterilized clean bench and sealed well. These initial cultures were placed in an incubator at 22 ℃ for 14 days until fungal mycelium covered the surface of the medium, as shown in Figure 3.1.



Ganoderma lucidum *Fomes fomentarius* *Trametes pubescens*

Ganoderma lucidum *Fomes fomentarius* *Trametes pubescens*

Figure 3.1 A) Day 1 of initial cultures, B) Day 14 of initial cultures.

3.2.2 Substrates Preparation

In this study, the initial culture of *Ganoderma lucidum* was inoculated on the rye grain before growing on the final substrate, while the other two strains skipped this step and were inoculated on the final substrate directly.

Rye grains (Yupik, Canada) were rinsed and cleaned before soaking in tap water for 12 hours (Shields, 2020). During soaking, 1.5 wt. % (dry weight percentage) gypsum was added to rye grains. The grains were then simmered for about 15-20 minutes to ensure they absorb sufficient water (Shields, 2020). After this, the excess water outside the grains was drained out. Finally, the grains were loaded into autoclavable filter patch bags (Grow Mushrooms Canada, Canada) with the dimensions of $8'' \times 5'' \times 19''$ and a 0.5-micron filter.

There are three types of final substrates. They are 100% hemp hurds (Natural fiber, Canada), 70 wt. % (wet weight percentage) hemp hurds mixed with 30 wt. % (wet weight percentage) waste paper pellets, and 60 wt. % (wet weight percentage) hemp hurds mixed with 40 wt. % (wet weight percentage) waste paper pellets. Hemp hurds were soaked in tap water for 12 hours and then were drained out for about 1 hour to remove excess water. For the hybrid substrates, the paper was hydrated with tap water and squeezed out excess water before adding to the wet hemp hurds that had been drained out. The hybrid substrates were mixed thoroughly by hand. All substrates were filled into autoclavable filter patch bags separately.

All the filter patch bags with substrates were sterilized in the autoclave at 121℃ and 15 psi pressure for 1 hour and cooled down to room temperature (21±2°C).

3.2.3 Fabrication Methods and Conditions

3.2.3.1 Grain cultures cultivation

Ganoderma lucidum was inoculated into cooled autoclavable bags filled with rye grains from Petri dishes. Autoclavable bags containing inoculated rye grains were placed in a dark incubator with controlled temperature and humidity. The rye grains were covered by white mycelium completely after being cultivated at 28℃ and 80% relative humidity (RH) for six days, as shown in Figure 3.2.

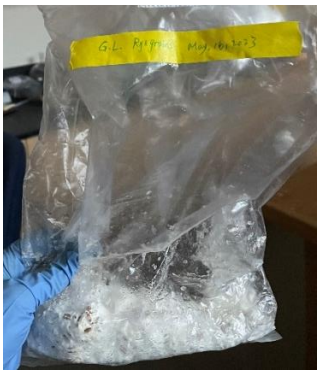


Figure 3.2 Grains inoculated with *Ganoderma lucidum* after 6 days

3.2.3.2 Final substrates inoculation

For *Ganoderma lucidum*, rye grain cultures were inoculated on the autoclaved and cooled final substrates after the 6-day incubation. In each bag of final substrates, 25 wt. % rye grain cultures were added and mixed thoroughly by hand from the outside of the bag.

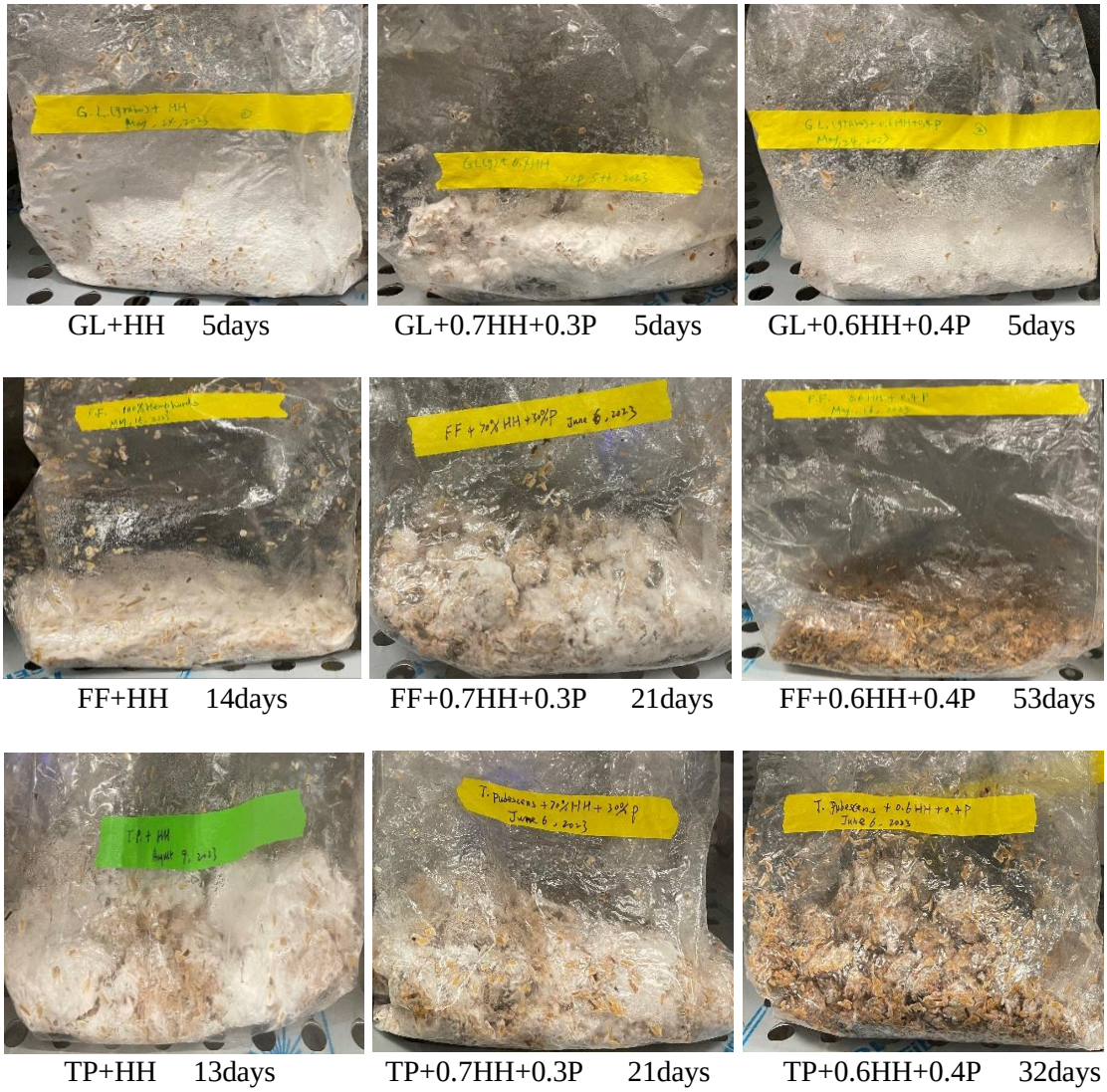
For *Fomes fomentarius* and *Trametes pubescens*, the initial cultures were inoculated on the final substrates directly from the Petri dish.

Bags with inoculated final substrates were put in a dark incubator with a temperature of 28℃ and RH of 80% for several days until substrates were covered by white mycelium completely, and the duration of the cultivation varies for each protocol, as shown in Figure 3.3. In this step, nine different protocols were made, as shown in Table 3.1. Samples made of pure hemp hurds were used as controls for each fungal species. The labels are coded using the initials of each fabrication variable. For instance, FF+0.7HH+0.3P represents *Fomes fomentarius* strains being inoculated on the hybrid substrates consisting of 70% hemp hurds and 30% waste paper. During the cultivation, the inoculum was shaken and smashed from the outside of the bag by hand every few days to ensure homogeneous cultivation and stimulate the mycelium growth.

Table 3.1 Labels of each protocol

Lable	Fungal species	Proportion of hemp hurds (%)	Proportion of paper (%)
GL+ HH	*Ganoderma lucidum*	100	0
GL+ 0.7HH+0.3P	*Ganoderma lucidum*	70	30
GL+ 0.6HH+0.4P	*Ganoderma lucidum*	60	40

FF+HH	*Fomes fomentarius*	100	0
FF+0.7HH+0.3P	*Fomes fomentarius*	70	30
FF+0.6HH+0.4P	*Fomes fomentarius*	60	40
TP+HH	*Trametes pubescens*	100	0
TP+0.7HH+0.3P	*Trametes pubescens*	70	30
TP+0.6HH+0.4P	*Trametes pubescens*	60	40



GL+HH 5days GL+0.7HH+0.3P 5days GL+0.6HH+0.4P 5days

FF+HH 14days FF+0.7HH+0.3P 21days FF+0.6HH+0.4P 53days

TP+HH 13days TP+0.7HH+0.3P 21days TP+0.6HH+0.4P 32days

Figure 3.3 Growth situation of each protocol. The days below each picture represent the duration of cultivation. The uppercase letters GL represent *Ganoderma lucidum*, FF represent *Fomes fomentarius*, and TP represent *Trametes pubescens.*

3.2.3.3 Cultivation in the mold

When the entire matrix in the autoclavable bag was entirely covered by mycelium, they were transferred to plastic containers (Plastico, China) with dimensions of 8.4cm×8.4cm×13.2cm. The sides and bottom of each container were pierced with needles, and the upper bottom was covered with plastic wrap, ensuring ventilation and moisture. In this step, the inoculum was filled in the containers for each protocol. Samples in containers were incubated at 28℃ and 80% RH for a while until they were covered in mycelium and grew into shape. Subsequently, samples were de-molded and put upside down in plastic baskets with a plastic bag sealing them, as shown in Figure 3.4. Sponges soaked in distilled water were put inside to maintain steady humidity levels in the basket between 80%-85% RH during the cultivation. The sealed baskets were placed in the incubator at 28℃ and 80% RH for several days until the surface of the sample was covered entirely by white mycelium.

Figure 3.4 Demolded samples in the sealed baskets and cultivated in the incubator

3.2.3.4 Dehydration

All samples were dried in the oven at 75℃ for 24 hours.

3.2.4 Morphological Analysis

All samples were analyzed by visual inspection and light microscope (SteREO Discovery.V8, Carl Zeiss Microscopy) from the top view and cross-section of samples.

3.2.5 Dry Density Measurement

Six samples were tested for dry density under each protocol. Weight measurements were conducted using a precision balance scale accurate to 0.01g, while dimensions were measured using a digital caliper.

The dry density was calculated using Equation (3-1).

$$d\ (kg/m^3) = \frac{m}{v} \qquad (3\text{-}1)$$

where,

d= dry density [kg/m^3], m= the mass of each sample after drying [g], v= the volume of each sample after drying[cm^3].

3.2.6 Moisture Exposure Testing

Three dried specimens were used to test water absorption under each protocol at 40℃ and RH of 60% and 80% (Appels et al., 2019). Specimens were put on the grid in a moisture chamber, as shown in Figure 5, ensuring each side of the sample was exposed to the selected temperature and RH. Before putting samples in the chamber, the thickness was measured at three different points of the sample using a digital caliper. The thickness of the three points was measured again after 4 and 96 hours. The weight of each specimen was measured before moisture exposure and every 10 minutes within the first hour and then measured after 2, 4, 24, 48, and 96 hours, using a precision balance scale accurate up to 0.01g.

Figure 3.5 Moisture exposure testing

3.2.7 Mechanical Testing

This study used the Standard Test Method for Compressive Properties of Rigid Cellular Plastics (ASTM D1621-16) for compressive testing. Three dried specimens were subjected to compressive testing under each protocol. The compressive properties of all the samples were tested by an

unconfined compression testing system (Wykeham Farrance, Slough, England) with a load cell at 2.5klb at ambient conditions (21℃ and about 50% RH). The crosshead displacement rate was 10% of the sample thickness per minute. The test was stopped at the compressive strain of 70%. Compressive strain ε is the length change per unit of original length along the longitudinal axis due to a compressive force. It is worth noting that the surface of the load cell could not make complete contact with the sample surface as the surface of the MBM is rough, leading to the inaccuracy of the initial testing data, which should be handled appropriately. The load-displacement curve, the result of the testing, was converted to the stress-strain diagram. The compressive stress (σ) and strain (ε) were calculated by Equations (3-2) and (3-3), respectively.

$$\sigma = \frac{F}{A}\ (MPa) \qquad (3\text{-}2)$$

and

$$\varepsilon = \frac{\Delta L}{L_0} \qquad (3\text{-}3)$$

where,

σ= compressive stress [MPa], F= compressive load [MPa], A= area of original cross-section of the sample [mm^2], ε= compressive strain [-], ΔL= displacement of loading surface [mm], L_0= the original thickness of the sample [mm].

The compressive strength (σ) and Young's modulus (Ey) were used as indexes to evaluate the compressive properties. Compressive strength reflects the ability of samples to withstand loads that tend to reduce size. The calculation method of compressive strength is shown in Equation (2). According to the Standard Test Method for Compressive Properties of Rigid Cellular Plastics (ASTM D1621-16), the compressive strength (σ) is the stress at a yield point if it occurs before 10% deformation or the stress at 10% strain when there is no such a yield point. In addition, some previous studies used compressive strength at 20% or 35% strain to demonstrate MBMs' mechanical properties (Pohl et al., 2022; Tacer-Caba et al., 2020). This study also chose the strength at 35% strain except for the definition of compressive strength in ASTM D1621-16 to depict the samples' compressive strength. Young's modulus is calculated as Equation (3-4), which measures the compressive stiffness of materials. It is defined as the ratio of the stress and the

resulting axial strain in the linear elastic region. Within this region, the material experiences elastic deformation, indicating that it can return to its original shape after the stress is removed.

$$E_y = \frac{\sigma}{\varepsilon} \qquad (3\text{-}4)$$

where,

E_y= Young's modulus [MPa], σ= compressive stress [MPa], ε= compressive strain [-].

3.2.8 Statistical analysis

The dry density, weight and thickness increase, compressive strength, and Young's modulus were statistically analyzed in Microsoft Excel by One-way analysis of variance (ANOVA) ($P \leq 0.05$).

3.3 Results and Discussion

3.3.1 Fabrication Duration

The fabrication process's time consumption varies among protocols. Table 3.2 presents the duration of each step and the total time consumption for every protocol. No data is available on samples made of *Fomes fomentarius* or *Trametes pubescens* cultivated on hybrid substrates containing 60% hemp hurds and 40% waste paper, as these two strains did not thrive on such substrates. Samples made of hybrid substrates exhibited longer production times than those from 100% hemp hurds.

Table 3.2 Time consumption of the fabrication process

Samples	Grow on substrates (days)	Grow in containers (days)	Demolded growth (days)	Dehydration (days)	Total (days)
GL+ HH	11	4	4	1	20
GL+ 0.7HH+0.3P	11	7	5	1	24
GL+ 0.6HH+0.4P	11	7	5	1	24
FF+HH	13	4	4	1	22
FF+0.7HH+0.3P	16	4	5	1	26
TP+HH	15	5	7	1	28
TP+0.7HH+0.3P	20	8	7	1	36

3.3.2 Morphology

MBMs of different protocols have different appearances, and all samples before and after dehydration are shown in Appendix 1 and 2. Figure 3.6. compares the appearance of samples after dehydration. The mycelium morphology and thickness on the samples' surface were observable via light microscopy in both top and cross-section views (Figure 3.7).

There was no apparent difference between mycelium morphology inside the samples fed with hybrid substrates and those fed with pure hemp hurds. However, there were differences in the surface mycelium skin of samples with different substrate types. Not every sample made of hybrid substrates had the same level of mycelium coverage as those fed with 100% hemp hurds. It can be seen that the mycelium skin on the surface of *Ganoderma lucidum*-based samples with hybrid substrates was not as dense as that on ones fed with pure hemp hurds. The same situation can be seen in *Trametes pubescens*-based samples. However, FF+0.7HH+0.3P displayed the most complete and puffy mycelium skins among all the samples fed with hybrid substrates.

Furthermore, only *Ganoderma lucidum* was able to grow on a mixture containing 60% hemp hurds and 40% paper, and a layer of mycelium skin covered the surface of samples, indicating *Ganoderma lucidum*'s robust adaptability. This suggests that strains' adaptability should be considered when using hybrid substrates with more paper.

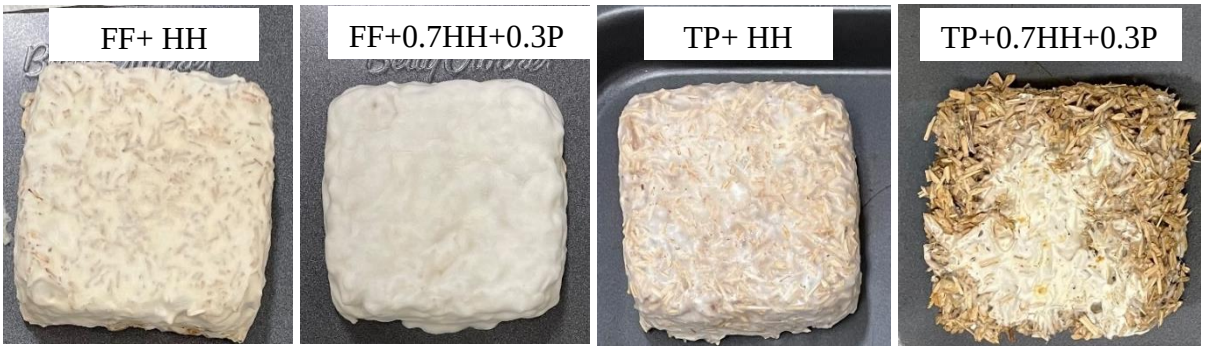
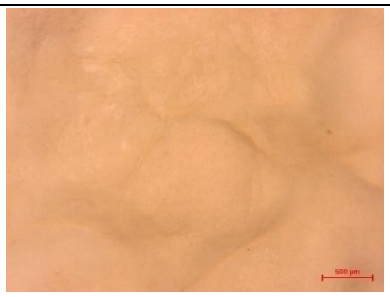
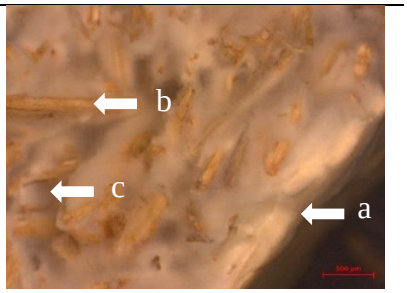
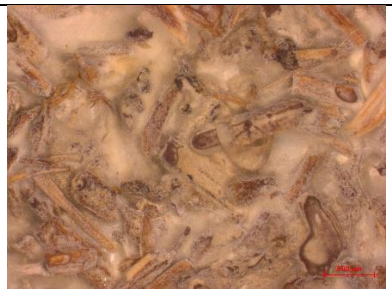
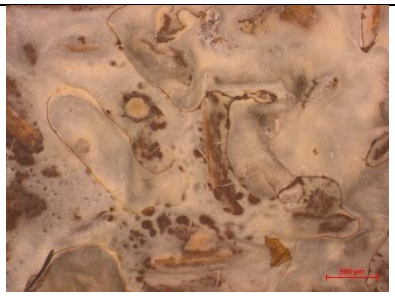


Figure 3.6 Appearance of MBMs of different protocols

Samples	Top view	Cross section
GL+ HH		
GL+ 0.7HH+0.3P		
GL+ 0.6HH+0.4P		

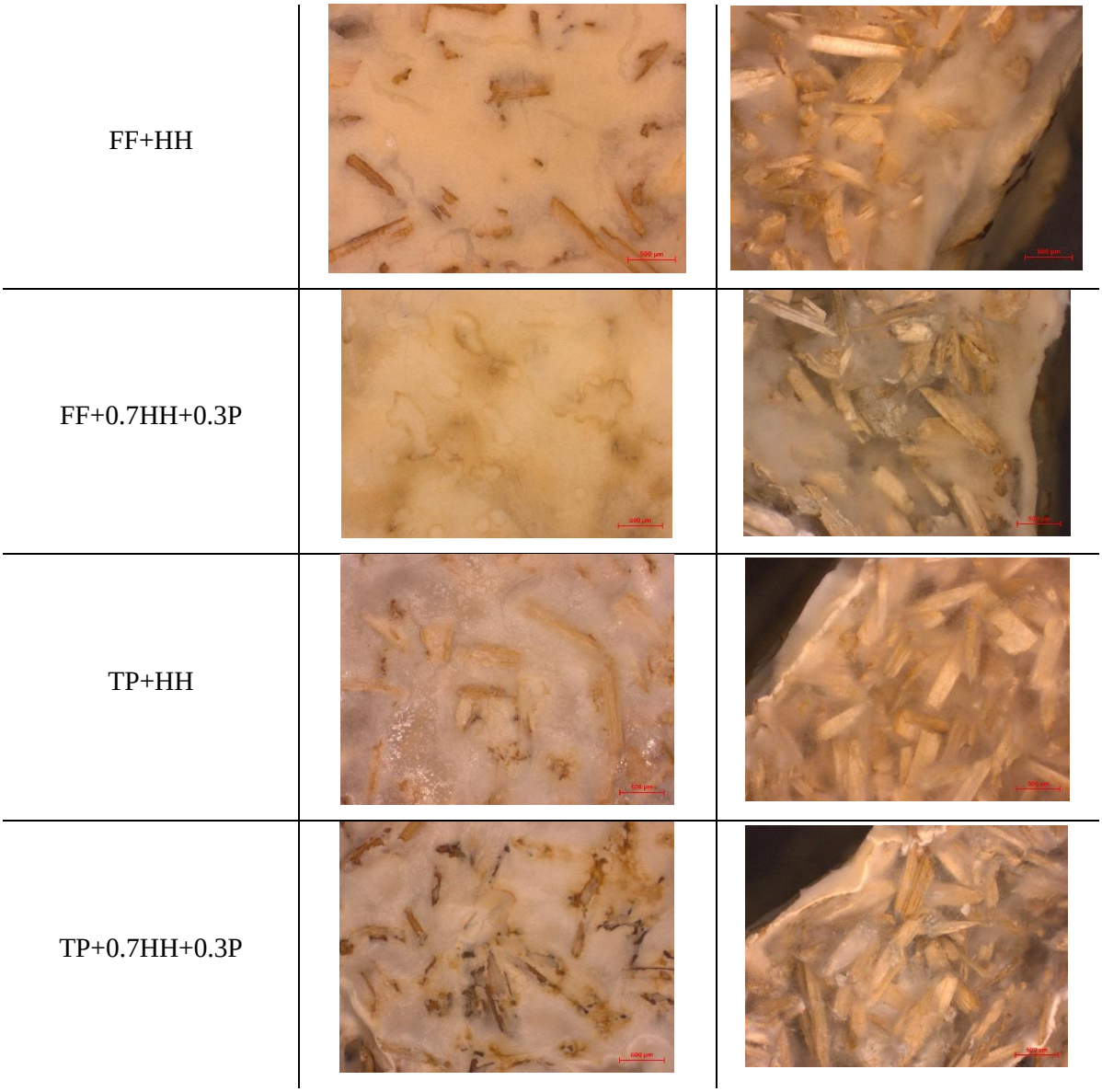


Figure 3.7 Light microscope images of all the samples from the top view and cross-section. Arrows indicate mycelium skins (a), substrates (b), and air voids (c). Scale bars represent 500μm.

3.3.3 Dry Density

Dry density is an important physical property for MBMs when used as packaging materials because it links to transportation costs and the convenience of application. In this study, the dry density of samples ranged from 0.070 to 0.145 g/cm^3 (Table 3.3), falling within the range of previous studies that is 0.025-0.954 g/cm^3 (Aiduang et al., 2022). There are statistically significant differences in dry densities between samples with hybrid substrates and pure hemp hurds ($p \leq 0.05$). Among samples with 30% paper in the substrates, FF+0.7HH+0.3P (0.097 g/cm^3) showed

the lowest value of density, followed by TP+0.7HH+0.3P (0.115g/cm^3) and GL-based samples (0.135g/cm^3). Samples GL+0.6HH+0.4P fed with 40% paper saw the highest dry density (0.145g/cm^3). Samples fed with hybrid substrates exhibited a higher density than those based on pure hemp hurds with the same fungal species. This aligns with a previous study, which showed that incorporating waste paper into the substrates can increase the dry density of MBMs, and the higher the paper ratio, the greater the density (Teeraphantuvat et al., 2024). In addition, the values of dry density varied among different strains in this study following the previous research results that mycelium strains also affect the dry density of MBMs (Aiduang et al., 2022; Girometta et al., 2019; Jones et al., 2020).

Although the density of all samples tested in this study is higher than traditional EPS packaging, which ranges from 0.012 to 0.05 g/cm^3 (British Plastics Federation, 2024), it is still lower than that of pulp molding packaging, which is a widely used eco-friendly protective packaging material with a density of 0.2-1.0 g/cm^3 (Debnath et al., 2022). Therefore, the samples created with hybrid substrates in this study hold promise for utilization in the packaging industry as an alternative to EPS, given their lower density than existing green packaging materials. This could lead to decreased transportation expenses.

Table 3.3 Overview of dry density (±SEM) of MBMs (ANOVA, $p \leq 0.05$).

Materials	Dry Density (g/cm^3)
FF+HH	0.073±0.001
FF+0.7HH+0.3P	0.097±0.002
TP+HH	0.070±0.001
TP+0.7HH+0.3P	0.115±0.004
GL+ HH	0.114±0.004
GL+ 0.7HH+0.3P	0.135±0.007
GL+ 0.6HH+0.4P	0.145±0.006

3.3.4 Moisture Exposure

Tables 3.4 and 3.5 show each sample's weight and thickness increase after moisture exposure testing.

Table 3.4 The increase of weight and thickness (±SEM) of samples after being exposed to 60% RH at 40℃ for 96 hours. Letters indicate statistically significant differences with corresponding materials (ANOVA, $p \leq 0.05$).

Materials	Weight increase (%)	Thickness increase (%)
FF+HH (a)	7.21±0.23 [b]	1.02±0.49
FF+0.7HH+0.3P (b)	5.68±0.10 [a]	1.46±0.43
TP+HH (c)	6.99±0.05 [d]	1.85±0.73
TP+0.7HH+0.3P (d)	5.56±0.23 [c]	0.64±1.01
GL+ HH (e)	4.56±0.14 [f,g]	0.84±0.32
GL+ 0.7HH+0.3P (f)	4.49±0.11 [e,g]	1.05±0.53
GL+ 0.6HH+0.4P (g)	2.93±0.12 [e,f]	1.57±0.69

Table 3.5 The increase of weight and thickness (±SEM) of samples after being exposed to 80% RH at 40℃ for 96 hours. Letters indicate statistically significant differences with corresponding materials (ANOVA, $p \leq 0.05$).

Materials	Weight increase (%)	Thickness increase (%)
FF+HH (a)	13.38±0.34 [b]	1.47±0.53
FF+0.7HH+0.3P (b)	15.42±0.45 [a]	1.44±1.16
TP+HH (c)	10.68±0.76	2.67±1.00 [d]
TP+0.7HH+0.3P (d)	11.48±0.20	-1.32±0.77 [c]
GL+ HH (e)	13.23±0.39 [f,g]	3.22±1.26
GL+ 0.7HH+0.3P (f)	14.69±0.08 [e,g]	2.46±0.69
GL+ 0.6HH+0.4P (g)	14.58±0.26 [e,f]	1.01±0.59

3.3.4.1 Weight increase

Figure 3.8 shows that the overall weight increase was higher in samples exposed to 80% RH than in those exposed to 60% RH. They showed a 10.68-15.42% and a 2.93-7.21% final weight increase at 40 ℃, respectively. The previous study linked the higher moisture uptake at 80% RH to the start of the capillary condensation (Kochumalayil & Berglund, 2014). In this study, all samples had a weight increase of below 8% at 60% RH and above 10% at 80% RH, as shown in Figure 8. At 60% RH, samples fed with hybrid substrates exhibited a 1.43-1.56% reduction in water absorption compared to those fed with pure substrates. Conversely, at 80% RH, the opposite trend was observed with samples fed with hybrid substrates exhibiting higher water absorption, but the

differences were slight at 0.8-2.04%. This implies that MBMs derived from hybrid substrates are well-suited for application in environments with lower RHs, such as 60%. In environments with a higher RH, like 80%, opting for MBMs fed with pure hemp hurds, which exhibited greater humidity resistance, would be more suitable.

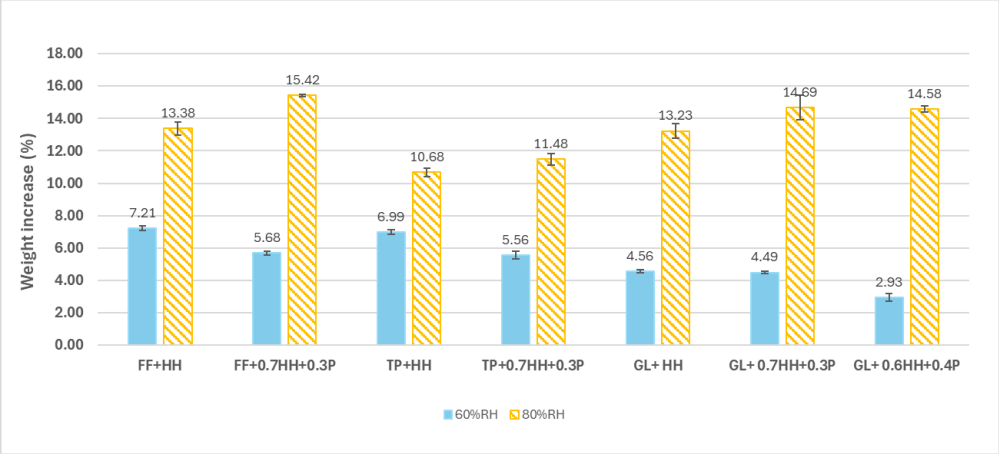


Figure 3.8 Weight increase percentage of samples after exposure to 60%RH and 80%RH at 40℃ for 96 hours

Figure 3.9 illustrates that samples comprising hybrid substrates exhibited a similar weight gain trend as those comprising pure hemp hurds at 60% RH. They gained water rapidly in the first ten minutes, and the weight leveled off after four hours of exposure, excluding samples GL+0.6HH+0.4P that absorbed moisture quickly in the first hour and gradually saturated after four hours. On the other hand, at 80% RH, samples FF+0.7HH+0.3P and TP+0.7HH+0.3P showed different weight-growing trends compared to those fed with pure hemp hurds after an hour of exposure. A closely resembling weight increase pattern was observed in *Ganoderma lucidum*-based samples fabricated from hybrid substrates compared to those from pure hemp hurds. Moisture absorption gradually increased until the fourth hour, followed by a notable surge between the fourth and twenty-fourth hour.

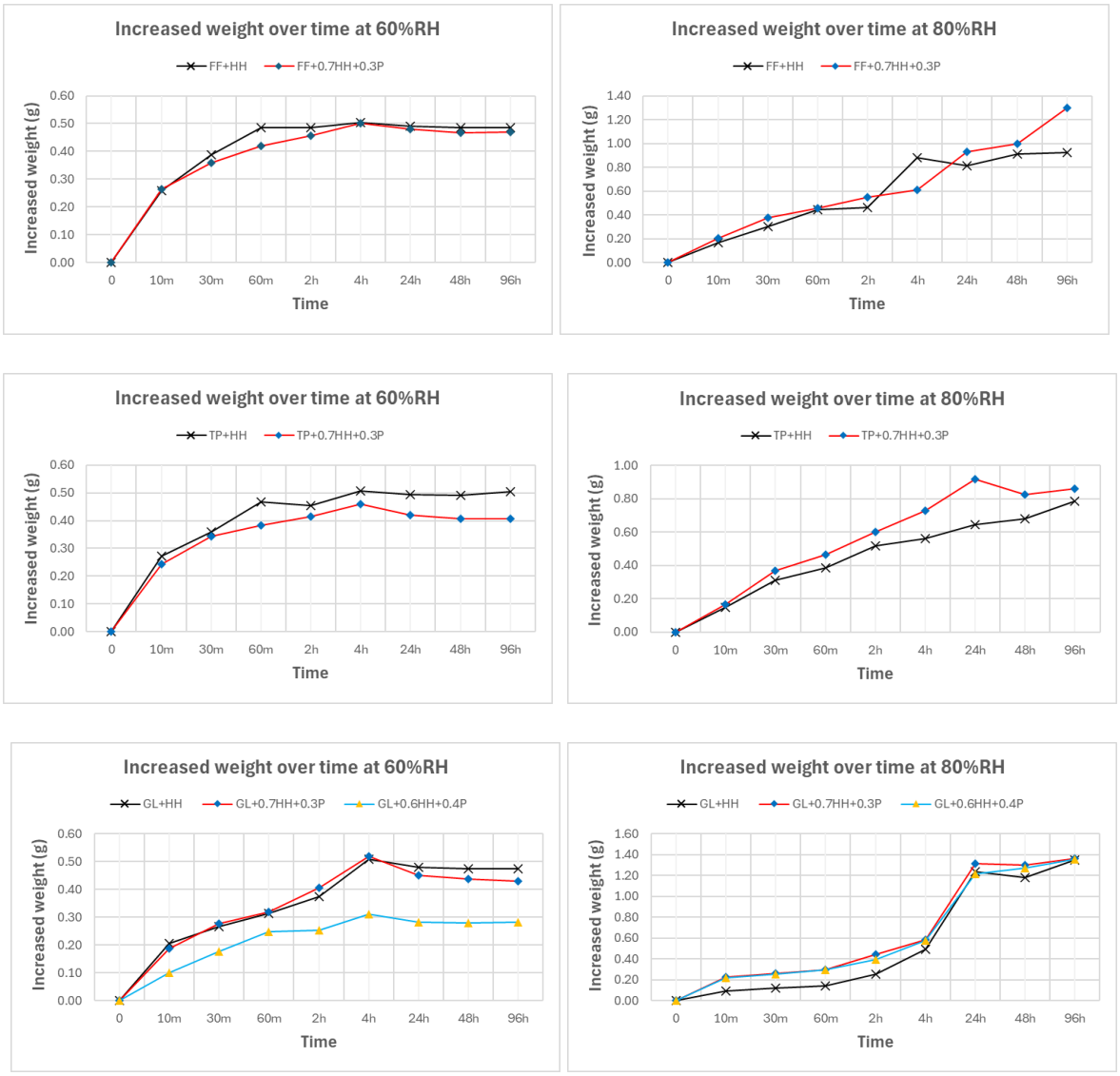


Figure 3.9 Increased weight (the weight measured at each time interval minus the original weight) at 60%RH and 80%RH.

3.3.4.2 Thickness increase

There are no statistically significant differences in thickness increase at 60% RH between samples with hybrid substrates and those with pure hemp hurds. The same situation can be seen at 80% RH, except for *Trametes pubescens*-based samples. In addition, the thickness increase of GL+0.6HH+0.4P and TP+0.7HH+0.3P was significantly lower at 80% RH than at 60% RH. This phenomenon aligns with a previous study, which attributed it to the materials collapse after water absorption (Appels et al., 2019). It is worth noting that *Trametes pubescens* did not grow well on the hybrid substrates, with little mycelium on the surface of samples compared to other samples

(Figure 3.6). This might lead to samples TP+0.7HH+0.3P collapsing easily and even decreasing in thickness at 80% RH.

By comparing samples' weight increase percentage and dry density (Figure 3.10), it can be observed that curves representing weight increase percentage are opposite to curves representing dry density at 60% RH. This suggests that a higher dry density would lead to a decrease in water absorption when exposed to an environment with 60% RH. However, this negative correlation between dry density and water absorption was not observed at 80% RH. This means that in a relatively lower humidity environment, adding paper to the substrates increases the density of MBMs but reduces the water absorption of materials. However, water reduction of MBMs with hybrid substrates can not always be seen in a higher-humidity environment.

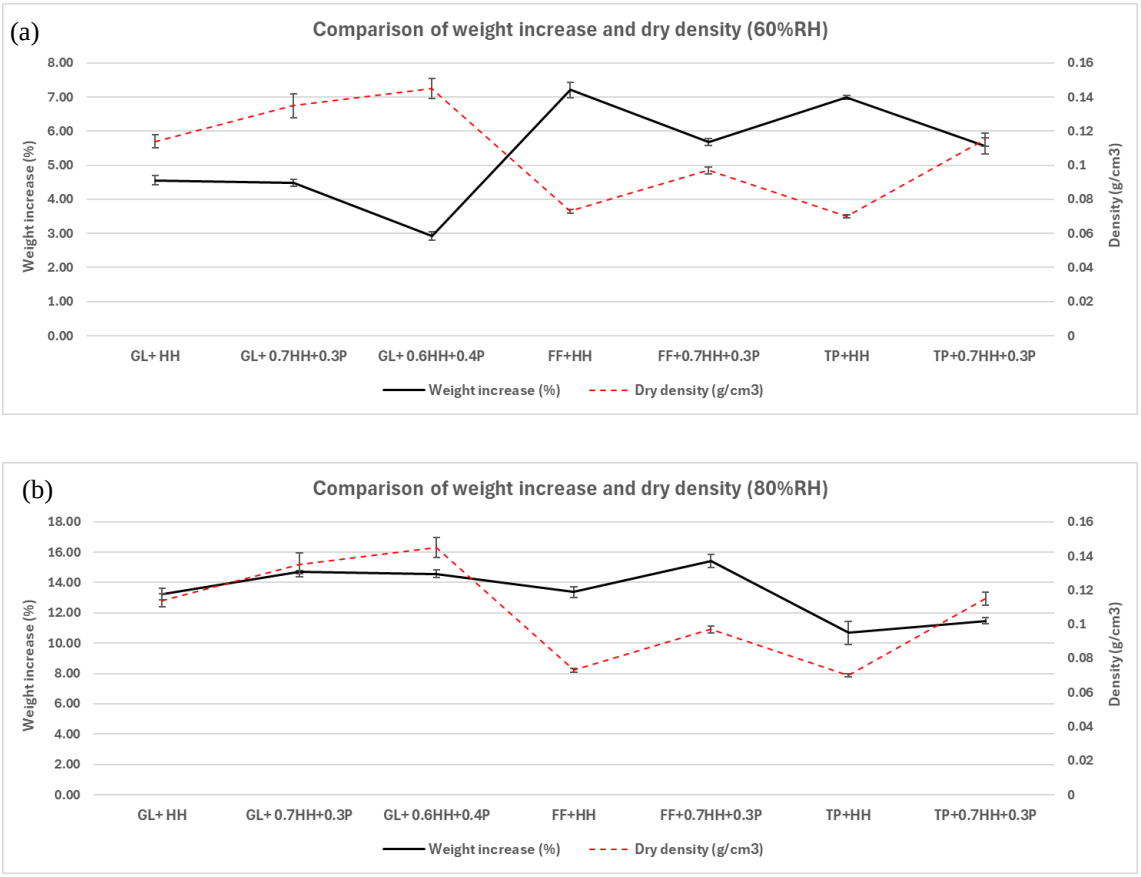


Figure 3.100 Comparison of weight increase percentage and dry density

Based on the discussion above, Table 3.6 lists the better protocol for each fungal species to produce MBMs with good water resistance properties at 60% RH and 80% RH, respectively.

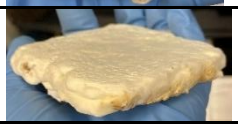
Table 3.6 Overview of samples with better water resistance properties

60% RH	Weight increase at 60% RH (%)	80%RH	Weight increase at 80% RH (%)
GL+0.6HH+0.4P	2.93	GL+HH	13.23
FF+0.7HH+0.3P	5.68	FF+HH	13.38
TP+0.7HH+0.3P	5.56	TP+HH	10.68

3.3.5 Mechanical Properties

3.3.5.1 Samples' appearance after compression

Samples of different protocols displayed different appearances and behaviors under compressive stress. Figure 3.11 illustrates the appearance of samples after undergoing compressive testing. All of the samples remained intact after compression, with no visible crack on the surface except for samples TP+ HH, which exhibited cracks on the side. Among samples fed with hybrid substrates, samples FF+0.7HH+0.3P showed the best integrity, with the mycelium skin covering the surface only appearing wrinkled but unbroken. It is worth noting that there was a little mycelium skin on the top and almost none on the side of samples TP+0.7HH+0.3P, so there is no issue of skin cracking for this protocol. In general, MBMs with hybrid substrates showed a good appearance after compressive testing.

Samples	TP+HH	TP+0.7HH+0.3P	FF+HH	FF+0.7HH+0.3P
Top view				
Flank view				
Samples	GL+HH	GL+0.7HH+0.3P	GL+0.6HH+0.4P	
Top view				

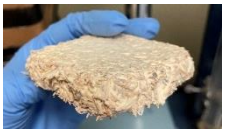
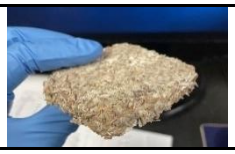
Flank view			

Figure 3.111 Top view and flank view of samples after compressive testing.

3.3.5.2 Compressive strength and Young's Modulus

Appendix 5 shows the original stress-strain curves of all samples. Except for TP-based samples, there are statistical differences in compressive strength values at 35% strain between samples with pure hemp hurds and hybrid substrates. Compressive strength at 35% strain was higher in samples with hybrid substrates than those with pure hemp hurds, except for samples GL+0.6HH+0.4P.

Compressive strength at 10% strain of samples based on hybrid substrates ranged from 0.028-0.083 MPa, partly falling within the EPS range from 0.040-0.345 MPa (Table 3.7). Compressive strength at 35% strain ranged from 0.136-0.320 MPa (Table 3.7), which is higher than the results of one previous study that ranged from 0.017-0.300 MPa (Tacer-Caba et al., 2020). The Compressive Young's modulus of MBMs with hybrid substrates varied between 0.282 and 0.770 MPa, which is lower than those of EPS, as shown in Table 7.

Table 3.7 Overview of compressive strength (±SEM) at 10% and 35% strain and Young's modulus (±SEM) of MBMs and EPS. Letters indicate statistically significant differences with corresponding materials (ANOVA, $p \leq 0.05$).

Materials	Compressive strength at 10% strain (MPa)	Compressive strength at 35% strain (MPa)	Young's modulus (Mpa)
FF+HH (a)	0.027±0.002	0.113±0.006 [b]	0.262±0.015
FF+0.7HH+0.3P (b)	0.028±0.001	0.142±0.002 [a]	0.282±0.009
TP+HH (c)	0.027±0.001	0.104±0.002	0.255±0.005
TP+0.7HH+0.3P (d)	0.033±0.004	0.136±0.013	0.324±0.033
GL+ HH (e)	0.072±0.003	0.281±0.002 [f,g]	0.687±0.024
GL+ 0.7HH+0.3P (f)	0.083±0.010	0.320±0.034 [e,g]	0.770±0.094
GL+ 0.6HH+0.4P (g)	0.055±0.007	0.206±0.021 [e,f]	0.529±0.063
EPS	0.040-0.345 (EPS12-EPS46)	-	5-40 (Matweb, n.d.)

	(ASTM International, 2021)		

The results presented above demonstrate that the compressive properties of MBMs for a specific mycelium strain depend on the substrate types, which is consistent with the findings of previous studies (Holt et al., 2012). Moreover, the outcomes demonstrate that adding 30% paper to the substrates can enhance the compressive properties. Compressive strength at 35% strain increased by 13.9% for GL-based samples, 25.7% for FF-based samples, and 30.8% for TP-based samples. The results that were obtained aligned with findings in previous studies that added paper can enhance MBMs' compressive properties (Solahuddin & Yahaya, 2021; Teeraphantuvat et al., 2024). This effect is because waste paper contains a large amount of cellulose fibers possessing high strength and stiffness, which can reinforce materials (de Oliveira et al., 2023). However, there may be a limit on the proportion of paper in the substrates beyond which the compressive properties of MBMs could decline. In this study, the compressive strength decreased when the paper content was increased to 40% in the substrates. Previous studies also showed that the compressive properties of materials would decline as the ratio of waste paper increased (Solahuddin & Yahaya, 2021; Teeraphantuvat et al., 2024). This phenomenon might be caused by an excessive amount of cellulose fibers in the substrates, which were difficult for strains to digest, leading to mycelium's inability to colonize substrates successfully (Teeraphantuvat et al., 2024). Therefore, finding the optimal ratio of waste paper in the substrates is worthy of further study.

3.3.5.3 Comparison of the dry density and compressive Young's Modulus

Figure 3.12 shows the correlation between Young's modulus and the dry density of samples. The results showed that the dry densities of MBMs do not always positively correlate with Young's modulus, which was in line with previous studies (Girometta et al., 2019). For samples GL+0.7HH+0.3P, FF+0.7HH+0.3P, and TP+0.7HH+0.3P, the compressive Young's modulus increased as dry density increased, which aligned with samples fed with pure hemp hurds. However, the lower Young's modulus of samples GL+0.6HH+0.4P resulted in the opposite relationship between Young's modulus and dry density compared to other samples.

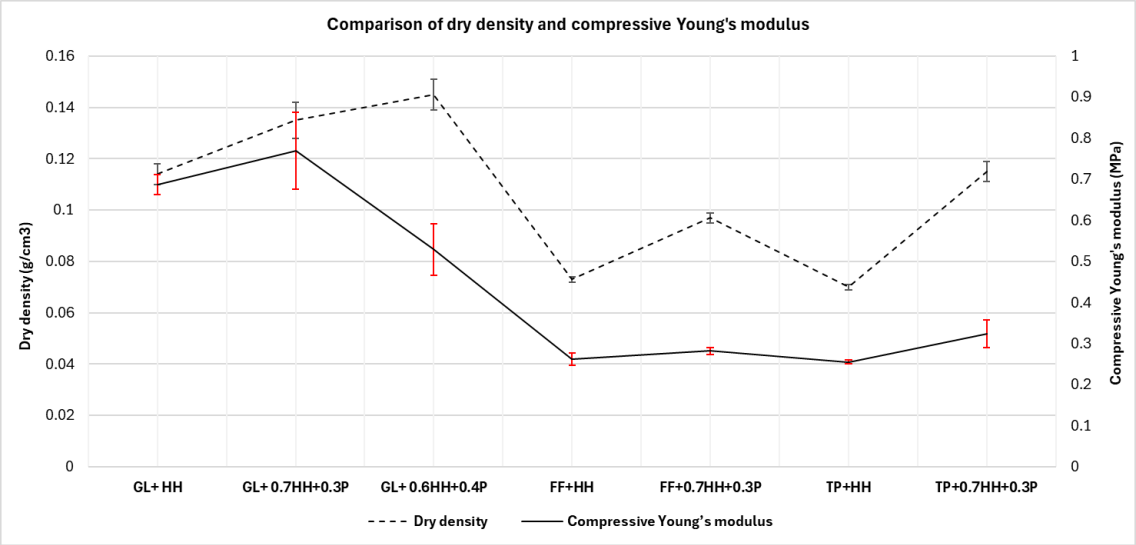


Figure 3.122 Comparison of dry density and compressive Young's modulus of samples

3.4 Conclusion

This study evaluated hybrid substrates of waste paper and hemp hurds for producing MBMs, including 70% hemp hurds+30% paper and 60% hemp hurds+40% paper. Only *Ganoderma lucidum* could grow on the mixture substrates with 40% paper successfully among the three fungal species used in the study. In addition, control samples made of pure hemp hurds were fabricated for each strain. Fabrication duration, morphology, dry density, water absorption, compressive strength, and Young's modulus were assessed on seven MBM protocols.

From the perspective of visual appearance, FF+0.7HH+0.3P had a velvety and foam-like appearance, GL+0.7HH+0.3P and GL+0.6HH+0.4P exhibited a more compact structure, and TP+0.7HH+0.3P showed a loose structure with less mycelium skin on the surface. Samples' dry densities increased along with the increase of the waste paper ratio in the substrates, indicating that mixing waste paper raises the density of MBMs. The dry density of samples with hybrid substrates also varied among different fungal species, with GL-based samples tending to have the highest density ($0.145 g/cm^3$) and FF-based samples having the lowest density ($0.097 g/cm^3$). This suggests that selecting suitable fungal species to grow on hybrid waste paper and hemp hurds substrates can offset part of the density increase due to hybrid substrates. The final weight increase was higher in samples exposed to 80% RH than in those exposed to 60% RH, with hybrid substrate-based samples showing an 11.48-15.42% and a 2.93-5.68% final weight increase at 40 ℃, respectively. Samples with hybrid substrates appeared to have better water resistance at 60% but

worse at 80% RH than those with pure hemp hurds, indicating the limitation of application conditions of MBMs made of hybrid substrates. Analysis of compressive properties revealed that samples with hybrid substrates had higher values than those fed with pure hemp hurds. This suggests that incorporating waste paper into the substrates can improve MBMs' compressive properties. However, excessive paper added to the substrates would lead to the decline of compressive properties.

Overall, this study upcycled waste paper to create MBMs, which showed enhancements in physical and mechanical properties compared to those made of pure hemp hurds. These improvements included a foam-like appearance, acceptable dry density, higher compressive properties, and better water resistance at 60%RH. In future studies, it would be valuable to investigate the mechanism behind the increased water absorption of MBMs with waste paper-based substrates at higher relative humidity and determine the optimal waste paper ratio in substrates to improve their properties. Furthermore, screening mycelium species capable of colonizing waste paper and exploring other low-value materials as potential substrates may further enhance MBMs' properties and reduce manufacturing costs.

Chapter 4 Investigation of the impacts of compaction on the properties of mycelium-based materials

Abstract

Mycelium-based materials (MBMs) are a new type of biomaterial made of mycelium and lignocellulosic wastes. Due to their environmental friendliness, they can potentially replace expanded polystyrene (EPS) as packaging materials. The physical and mechanical properties of MBMs are essential for their application in the packaging industry. This study assessed the effects of compaction on MBMs' characteristics. Three mycelium strains and three types of substrates were utilized to produce MBMs using different protocols. There are both compacted samples and uncompacted samples for each protocol. Dry density, water absorption, and compressive properties were compared between MBMs with compaction and those without compaction. Compacted samples in this study showed a 9.57-34.29% increase in dry density, a 28.57-129.63% increase in compressive strength at 10% strain, and a 37.32-139.42% increase at 35% strain, and a 27.66-142.35% increase in compressive Young's modulus. The impact of compaction on the water absorption of samples varied depending on the samples' recipes in this study.

Keywords: mycelium-based biomaterials; compaction; properties

4.1 Introduction

Polystyrene foam has been widely used as protective packaging, but its use is now being phased out worldwide due to its negative environmental impacts. As a result, academia and industry are actively seeking alternative packaging materials that are more sustainable and eco-friendly.

MBMs, a novel biomaterial that has emerged in recent decades, can potentially replace EPS. Composed of mycelium and agricultural lignocellulosic wastes, they undergo a unique manufacturing process. The mycelium acts as a "pipeline system", infiltrating the substrate and acting as a natural adhesive. This process releases enzymes that break down the substrate, binding the waste materials to the network of hyphae that comprise the individual strands of the expanding mycelium. The end product can be a bio-foam-like material suitable for packaging materials. The physical and mechanical properties of MBMs are significantly important for their application in the packaging industry.

Previous studies have focused on the impacts of fungal species and substrates on the properties of MBMs. Some research has shown that fungal species could affect MBMs' density and mechanical performance. For instance, MBMs fed with Agaricus bisporus and rapeseed cake had a higher density than that of MBMs produced from Ganoderma lucidum with the same substrate (Tacer-Caba et al., 2020). In addition, the cell wall composition of mycelium strains plays an important role in the MBMs' performance, especially the presence of chitin, proteins, lipids, and polysaccharides. Chitin helps aggregate particles of substrates, providing mechanical strength and reducing crack formation during compression (Teixeira et al., 2018; Yang et al., 2017). Proteins and lipids in the cell wall might act as plasticizers, while polysaccharides can offer stiffness to MBMs (Haneef et al., 2017). As for the substrates, their nutritional content and physical and mechanical properties would affect MBMs' properties. A previous study found that MBMs fed with *G. lucidum* or *P. ostreatus* and cellulose substrates presented higher Young's modulus and lower elongation than those fed with the same strains and dextrose-containing substrates because mycelium needs to synthesize more chitin to penetrate substrates when being fed with pure cellulose, which is more difficult to hydrolyze (Haneef et al., 2017). Another research has shown that the physical form of agricultural by-product fibers can exert an influence (Elsacker et al., 2019). For instance, using chopped hemp ($< 5mm$) and chopped flax ($< 5mm$) leads to higher compressive stiffness values of 0.77MPa and 1.18MPa, respectively, compared to loose hemp and loose flax, which have compressive stiffness values of 0.51MPa and 0.28MPa, respectively (Elsacker et al., 2019). In addition, researchers also found that particulate substrates, such as sawdust, provide higher compressive properties to the MBMs than fibrous substrates, such as straw (M. Jones et al., 2020).

Furthermore, fabrication methods also affect MBMs' properties. This study aimed to examine the effects of applying pressure before cultivation on the properties of MBMs using different recipes. There is a previous study looking at MBMs' properties under various protocols and packaging methods found that samples that were densely packed before cultivation demonstrated higher values in dry density, shear moduli, Young's moduli, and compressive moduli compared to those that were loosely packed (Yang et al., 2017). However, the previous study only used one mycelium strain and one type of substrate. In this study, three mycelium strains and three types of substrates were utilized to evaluate the impact of compaction on the density, water absorption, and

compressive properties of MBMs, aiming to provide a more comprehensive understanding of the role of compaction.

4.2 Materials and Methods

4.2.1 Initial Fungal Cultures

The fungal species *Ganoderma lucidum*, *Fomes fomentarius*, and *Trametes pubescens* were obtained from the Department of Microbiology at the University of Manitoba (see Chapter 3 for more information on the cultivation of initial fungi).

4.2.2 Substrates Preparation

As in Chapter 3, the initial culture of *Ganoderma lucidum* grew on the rye grain before inoculating on the final substrates, while the other two strains were inoculated on the final substrate directly. Preparing rye grains (Yupik, Canada) refers to section 3.2.2. Three types of final substrates: 100% hemp hurds (Natural fiber, Canada), 70 wt. % (wet weight percentage) hemp hurds mixed with 30 wt. % (wet weight percentage) waste paper pellets, and 100% rice husks. The preparation process is shown in Figure 4.1. All the filter patch bags with substrates were sterilized in the autoclave at 121℃ and 15 psi pressure for 1 hour and cooled to room temperature (21±2°C).

4.2.3 Fabrication Methods and Conditions

4.2.3.1 Grain cultures cultivation

Grain culture cultivation refers to section 3.2.3.1.

4.2.3.2 Final substrates inoculation

The method of inoculating *Ganoderma lucidum*, *Fomes fomentarius*, and *Trametes pubescens* on the final substrates was described in section 3.2.3.2.

The incubation method of inoculated substrates in bags was the same as described in section 3.2.3.2, and the duration of the cultivation is shown in Figure 4.2. Nine different protocols were made in this step, as shown in Table 4.1.

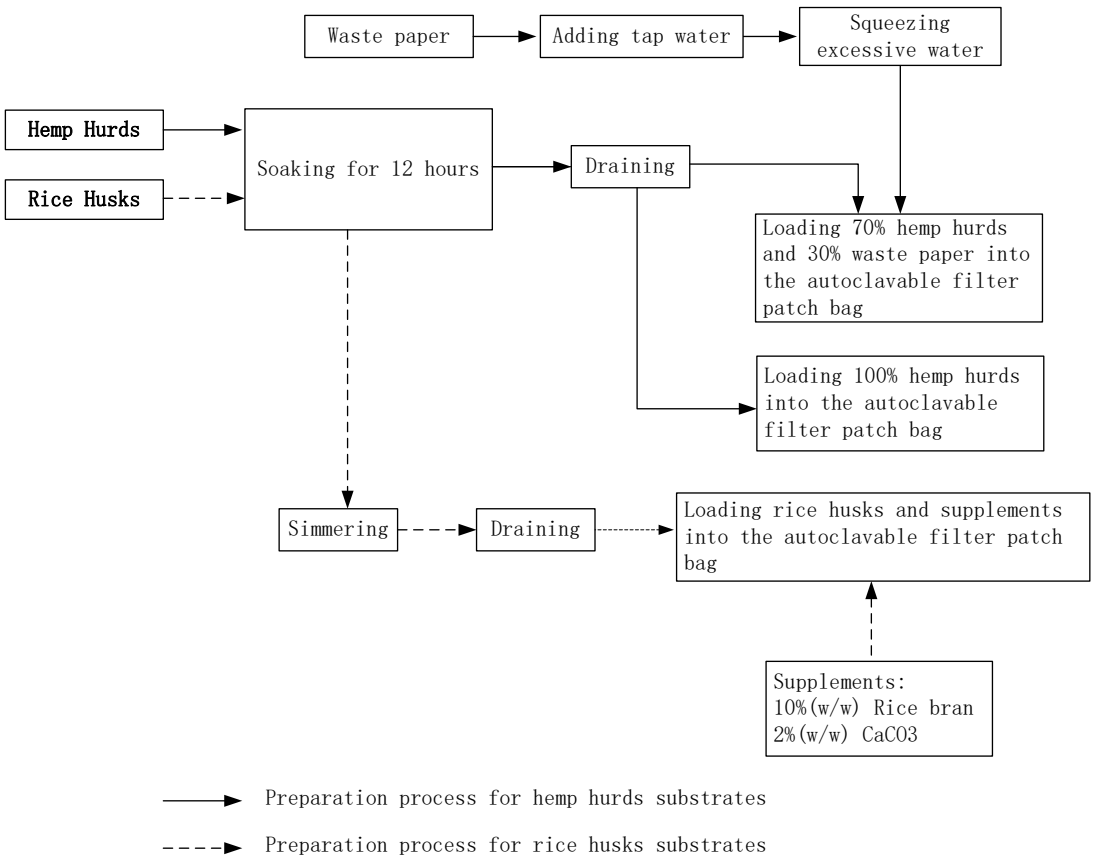


Figure 4.1 Preparation process for substrates.

The meaning of labels in Figure 4.2 and Table 4.1 is the same as those in section 3.2.3.2.

Table 4.1 Labels of each protocol

Lable	Fungal species	Proportion of hemp hurds (%)	Proportion of paper (%)	Proportion of rice husks (%)
FF+HH	*Fomes fomentarius*	100	0	0
FF+0.7HH+0.3P	*Fomes fomentarius*	70	30	0
FF+RH	*Fomes fomentarius*	0	0	100
TP+HH	*Trametes pubescens*	100	0	0
TP+0.7HH+0.3P	*Trametes pubescens*	70	30	0
TP+RH	*Trametes pubescens*	0	0	100
GL+ HH	*Ganoderma lucidum*	100	0	0
GL+0.7HH+0.3P	*Ganoderma lucidum*	70	30	0
GL+RH	*Ganoderma lucidum*	0	0	100

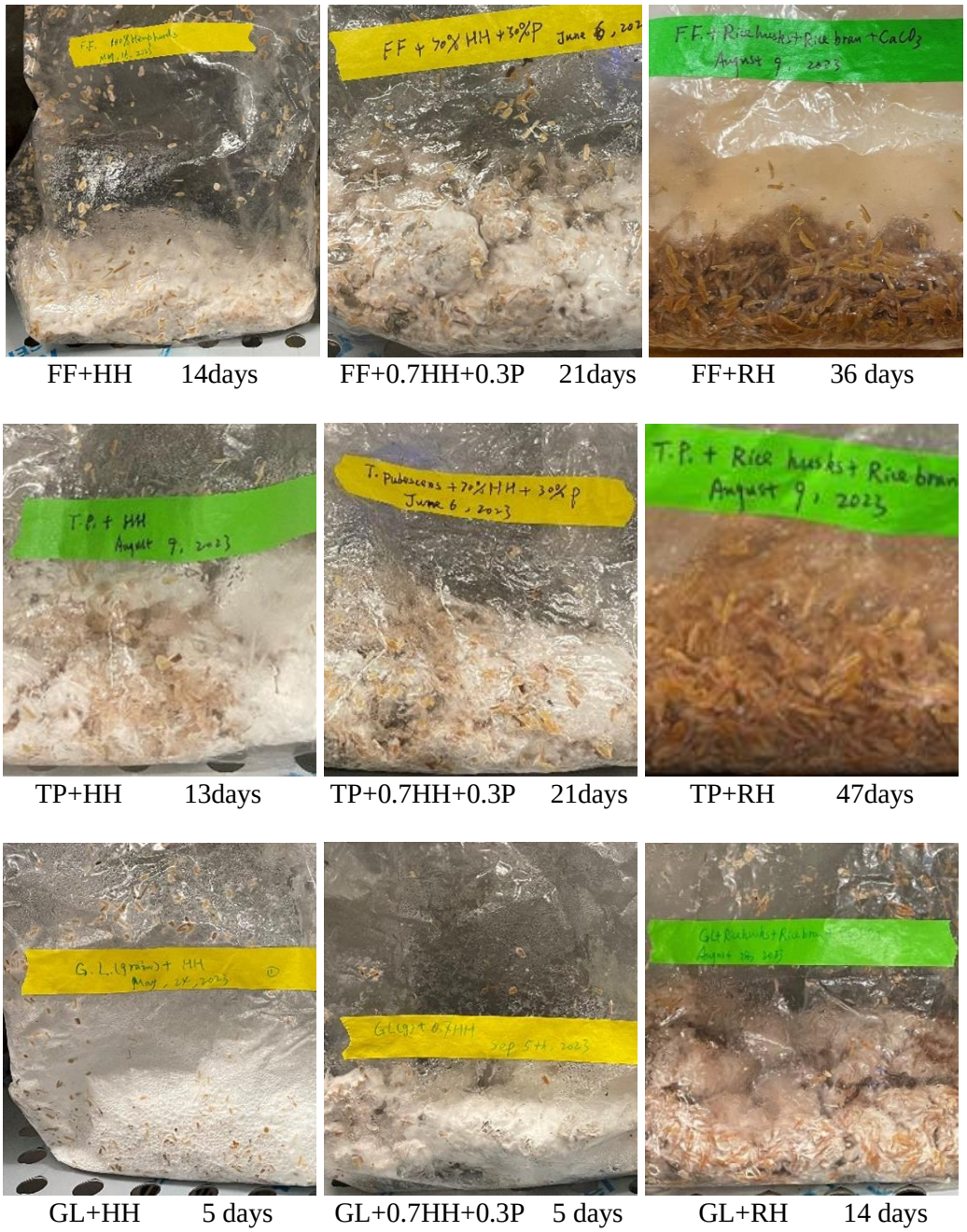


Figure 4.2 Growth situation of each protocol. The days on each picture represent the duration of cultivation.

4.2.3.3 Cultivation in the mold

When the entire matrix in the autoclavable bag was wholly covered by mycelium, they were transferred to plastic containers (Plastico, China) with dimensions of 8.4cm×8.4cm×13.2cm. The sides and bottom of each container were pierced with needles, and the upper bottom was covered with plastic wrap, ensuring ventilation and moisture.

In this step, there were two ways to fill the containers with inoculum from bags: 40g and 55g of inoculum for each protocol separately. This resulted in two different initial densities for each protocol, as two different masses of inoculum were filled into the same volume. For 40g inoculum, the material was put in the container by hand without any pressure during the filling process. For 55g inoculum, the material was pressed into the container by hand. In the following text, the number 40 in the samples' names represents uncompacted samples, and the number 55 in the samples' names represents compacted samples.

The cultivation environment and process are described in section 3.2.3.3.

4.2.3.4 Dehydration

All samples were dried in the oven at 75°C for 24 hours.

4.2.4 Morphological Analysis

All samples were analyzed by visual inspection and light microscope (SteREO Discovery.V8, Carl Zeiss Microscopy) from the top view and cross-section of samples.

4.2.5 Dry Density Measurement

The dry density measurement is described in section 3.2.5.

4.2.6 Moisture Exposure Testing

The moisture exposure testing method is described in section 3.2.6.

4.2.7 Mechanical Testing

The mechanical testing method is described in section 3.2.7.

4.2.8 Statistical analysis

The dry density, weight and thickness increase, compressive strength, and Young's modulus were statistically analyzed in Microsoft Excel by One-way analysis of variance (ANOVA) ($P \leq 0.05$).

4.3 Results and Discussion

4.3.1 Fabrication Duration

The fabrication process's time consumption varies among protocols. Table 4.2 presents the duration of each step and the total time consumption for each protocol. No data is available on

samples made of *Fomes fomentarius* or *Trametes pubescens* cultivated on rice husks, as these two strains did not thrive on rice husks in this study. It can be seen that samples subjected to compaction had a similar cultivation duration as those without compaction. The FF+0.7HH+0.3P-55 and GL+RH-55 samples required less cultivation time than those without compaction. It suggests that compaction may accelerate the mycelium extension rate on the sample surfaces because each step of fabrication, besides dehydration, ended when the material surface was completely covered by mycelium.

Table 4.2 The fabrication process's time consumption. 40 in the samples' name refers to uncompacted samples, and 55 refers to compacted samples.

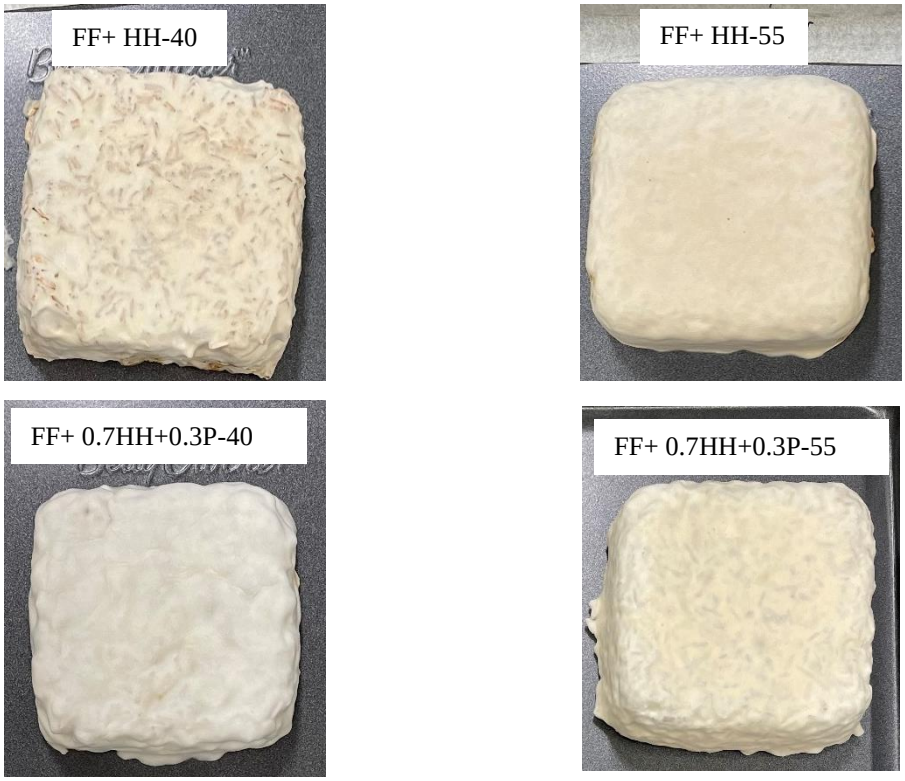
Samples	Grow on substrates (days)	Grow in containers (days)	Demolded growth (days)	Dehydration (days)	Total time (days)
Fomes fomentarius					
FF+HH-40	13	4	4	1	22
FF+HH-55	13	4	4	1	22
FF+0.7HH+0.3P-40	16	4	5	1	26
FF+0.7HH+0.3P-55	13	3	3	1	20
Trametes pubescens					
TP+HH-40	15	5	7	1	28
TP+HH-55	15	5	8	1	29
TP+0.7HH+0.3P-40	20	8	7	1	36
TP+0.7HH+0.3P-55	20	8	7	1	36
Ganoderma lucidum					
GL+ HH-40	11	4	4	1	20
GL+ HH-55	11	4	4	1	20
GL+ 0.7HH+0.3P-40	11	7	5	1	24
GL+ 0.7HH+0.3P-55	11	7	5	1	24
GL+RH-40	14	9	11	1	35
GL+RH-55	14	8	6	1	29

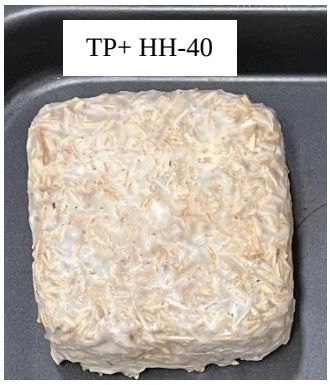
4.3.2 Morphology

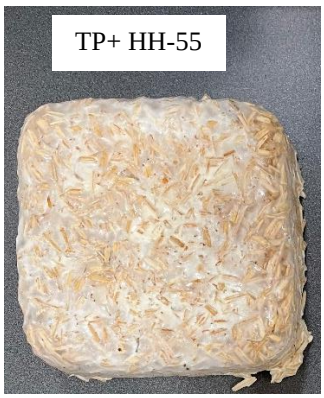
The appearance of all samples before and after dehydration is shown in Appendix 1-4. Figure 4.3 shows the impacts of compaction on the appearance of the final products. In addition, the

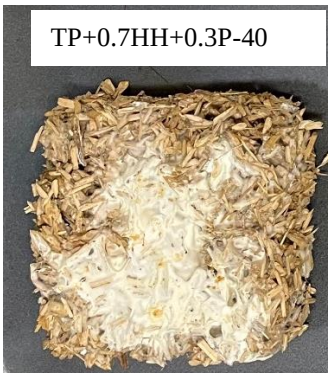
mycelium morphology and thickness on the samples' surface were observable via light microscopy in both top and cross-section views (Figure 4.4).

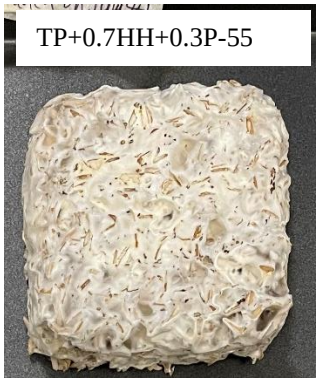
The visual appearance and the top view of the microscope show that compacted samples had similar or thicker mycelium skins on the surface than those without compaction. However, compacted samples GL+RH-55 and TP+0.7HH+0.3P-55 had significantly less mycelium inside than uncompacted ones. This may result from the combined effect of challenging substrates and insufficient air circulation inside the compacted samples. Insufficient air circulation inside compacted samples makes it difficult for hyphae inside samples to grow. Besides this, *Tramates pubescens* struggled to grow on hybrid substrates, and rice husk contains inhibitory substances for mycelium growth, such as momilactone A (MLA) (Hanai et al., 2005), making it difficult for *Ganoderma lucidum* to conolize.




TP+ HH-40

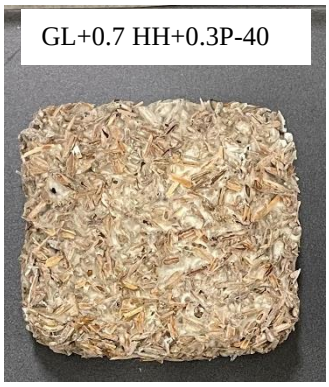

TP+ HH-55


TP+0.7HH+0.3P-40


TP+0.7HH+0.3P-55


GL+ HH-40


GL+ HH-55


GL+0.7 HH+0.3P-40

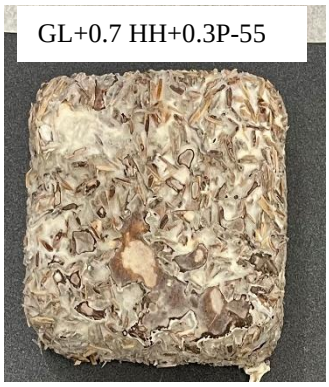
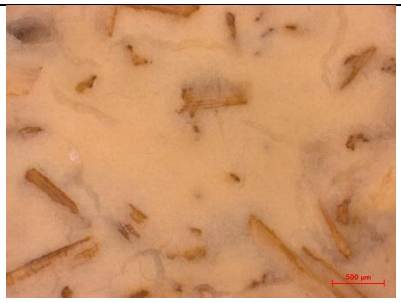
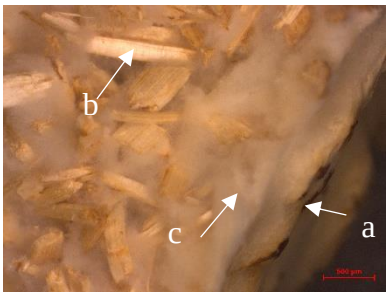
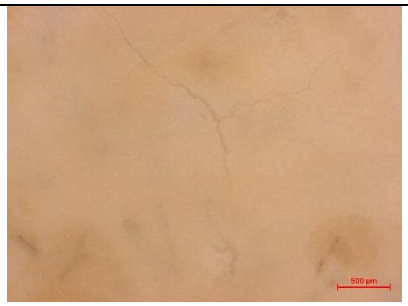
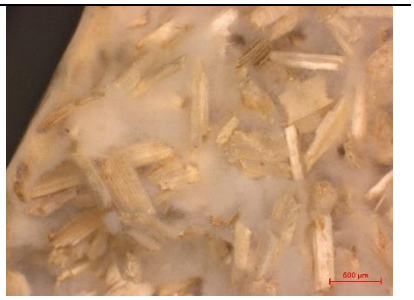
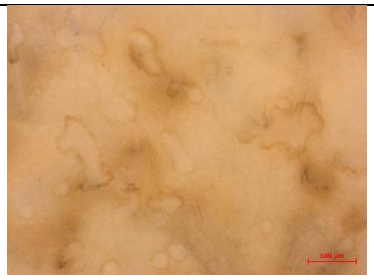
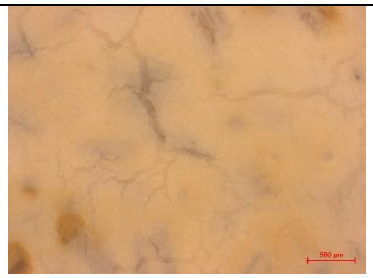
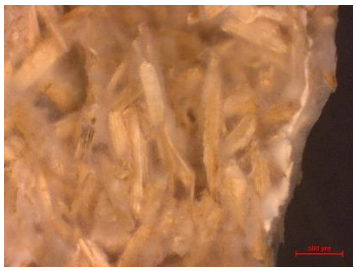
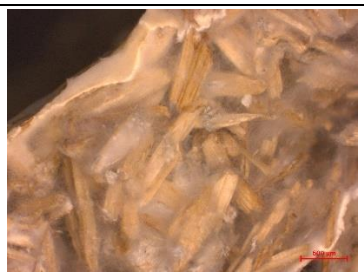
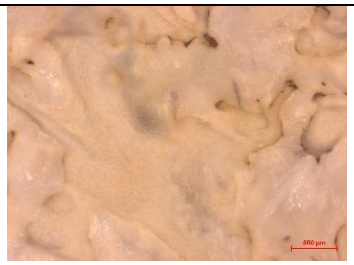
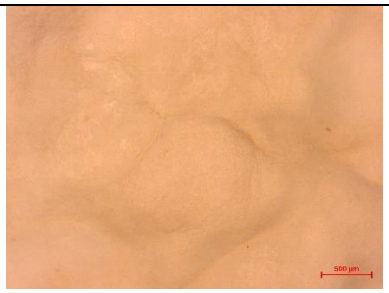
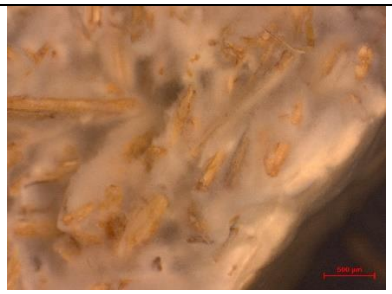
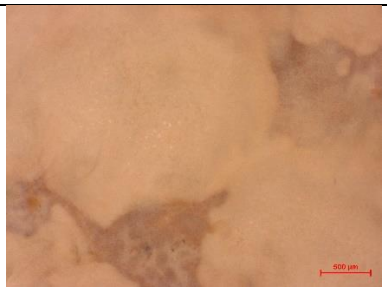

GL+0.7 HH+0.3P-55

Figure 4.3 Comparison of appearance between compacted samples and uncompacted samples. The number 40 on the picture's label represents uncompacted samples, and 55 represents compacted samples.

Samples	Top view	Cross section
FF+HH-40		
FF+HH-55		
FF+0.7HH+0.3P-40		
FF+0.7HH+0.3P-55		
TP+HH-40		

TP+HH-55		
TP+0.7HH+0.3P-40		
TP+0.7HH+0.3P-55		
GL+ HH-40		
GL+ HH-55		

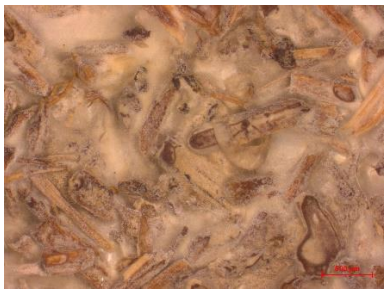
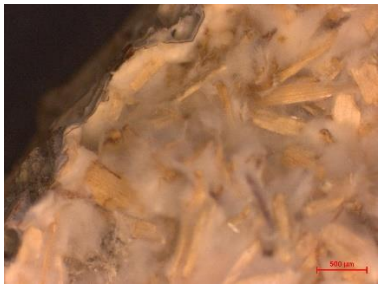
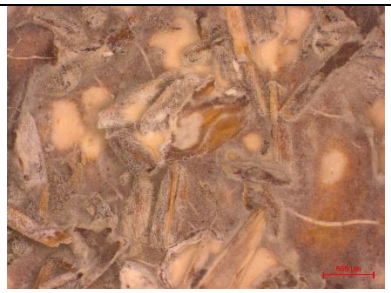
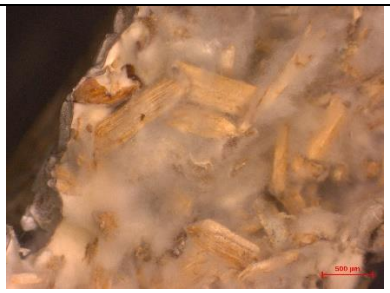
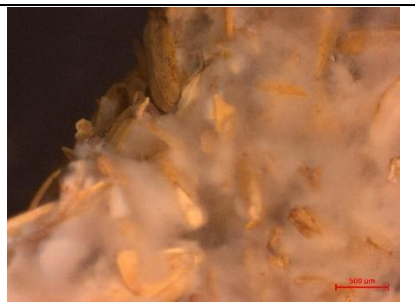
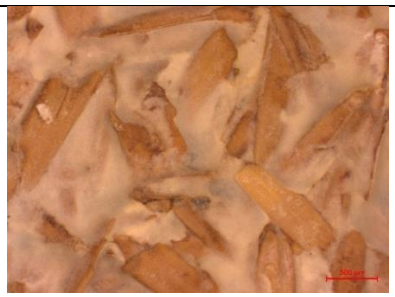
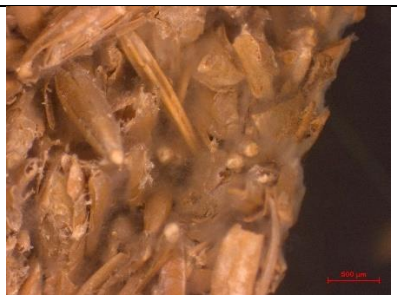
GL+ 0.7HH+0.3P-40		
GL+ 0.7HH+0.3P-55		
GL+RH-40		
GL+RH-55		

Figure 4.4 Light microscope images of all the samples from the top view and cross-section. Arrows indicate mycelium skins (a), substrates (b), and hyphae (c). Scale bars represent 500µm. The number 40 on the picture's label represents uncompacted samples, and 55 represents compacted samples.

4.3.3 Dry density

Dry density is an important physical property for MBMs used as packaging materials because it affects transportation costs and convenience in application. In this study, compacted samples' density was higher than uncompacted ones, with 0.089 to 0.163 g/cm^3 and 0.070 to 0.135 g/cm^3, respectively (Table 4.3), increasing from 9.57-34.29%. The density of all samples fell within the range of the previous study results, which is 0.025-0.954 g/cm^3 (Aiduang et al., 2022). There are

statistically significant differences in dry densities between compacted samples and uncompacted ones ($p \leq 0.05$).

The density of all samples tested in this study is higher than traditional EPS packaging, which ranges from 0.012 to 0.05 g/cm^3 (British Plastics Federation, 2024), but it is lower than that of pulp molding packaging, which is a widely used eco-friendly protective packaging material with a density of 0.2-1.0 g/cm^3 (Debnath et al., 2022). Therefore, the samples generated in this study could potentially serve as packaging materials to substitute EPS due to their lower density compared to existing green packaging materials.

Table 4.3 Overview of dry density (±SEM) of MBMs (ANOVA, p≤0.05).

Materials	Dry Density of uncompacted samples (g/cm^3)	Dry Density of compacted samples (g/cm^3)
Fomes fomentarius		
FF+HH	0.073±0.001	0.089±0.001
FF+0.7HH+0.3	0.097±0.002	0.121±0.005
Trametes pubescens		
TP+HH	0.070±0.001	0.094±0.001
TP+0.7HH+0.3P	0.115±0.004	0.126±0.004
Ganoderma lucidum		
GL+ HH	0.114±0.004	0.139±0.004
GL+ 0.7HH+0.3P	0.135±0.007	0.163±0.001
GL+RH	0.113±0.002	0.151±0.003

4.3.4 Moisture Exposure

The Water absorption property affects the application scenarios of MBMs. Tables 4.4 and 4.5 display each sample's weight and thickness increase after moisture exposure testing in this study.

The overall weight increase was higher in samples exposed to 80% RH than in those exposed to 60% RH. At 60% RH, the weight gain ranged from 4.49% to 7.21% and 4.59% to 6.98%for samples without compaction and with compaction, respectively. At 80% RH, the weight increase of uncompacted and compacted samples was 10.68- 15.42% and 11.02-19.04%, respectively. The compaction method did not significantly affect the water absorption properties of MBMs,

especially at 60% RH. Statistical analysis showed significant differences in weight increase between the compacted and uncompacted samples can only be seen in samples TP+HH, TP+0.7HH+0.3P at 60% RH, and samples FF+0.7HH+0.3P, TP+0.7HH+0.3P, GL+ HH, GL+0.7HH+0.3P, and GL+RH at 80% RH. The result shows that compaction did not necessarily increase or decrease the water absorption of MBMs, depending on the samples' recipes.

At 60%RH, the thickness increase of uncompacted and compacted samples was 0.64-2.55% and 0.09-2.02%, respectively. At 80% RH, the thickness increase of uncompacted and compacted samples was -1.32-3.22% and -0.24-3.24%, respectively. The negative value of thickness increase may be caused by material collapse (Appels et al., 2019).

Table 4.4 The increase of weight and thickness (±SEM) of samples after being exposed to 60% RH at 40℃ for 96 hours. Asterisks in the same row of the table indicate statistically significant differences between corresponding materials in weight increase or thickness increase (ANOVA, $p \leq 0.05$).

Materials	Weight increase of uncompacted samples (%)	Weight increase of compacted samples (%)	Thickness increase of uncompacted samples (%)	Thickness increase of compacted samples (%)
Fomes fomentarius				
FF+HH	7.21±0.23	6.98±0.10	1.02±0.49	2.01±0.34
FF+0.7HH+0.3	5.68±0.10	5.61±0.13	1.46±0.43	1.62±0.39
Trametes pubescens				
TP+HH	6.99±0.05 *	6.70±0.08 *	1.85±0.73	2.29±0.47
TP+0.7HH+0.3P	5.56±0.23 *	6.51±0.11 *	0.64±1.01	1.14±0.52
Ganoderma lucidum				
GL+ HH	4.56±0.14	4.59±0.06	0.84±0.32	0.80±0.44
GL+ 0.7HH+0.3P	4.49±0.11	4.59±0.51	1.05±0.53	2.02±0.75
GL+ RH	6.05±0.05	5.71±0.17	2.55±0.46 *	0.09±0.4 *

Table 4.5 The increase of weight and thickness (±SEM) of samples after being exposed to 80% RH at 40℃ for 96 hours. Asterisks in the same row of the table indicate statistically significant differences between corresponding materials in weight increase or thickness increase (ANOVA, $p \leq 0.05$).

Materials	Weight increase of uncompacted samples (%)	Weight increase of compacted samples (%)	Thickness increase of uncompacted samples (%)	Thickness increase of compacted samples (%)
Fomes fomentarius				
FF+HH	13.38±0.34	14.30±0.98	1.47±0.53	1.42±0.76
FF+0.7HH+0.3P	15.42±0.45 *	11.02±0.64 *	1.44±1.16	0.58±0.47
Trametes pubescens				
TP+HH	10.68±0.76	12.08±0.05	2.67±1.00	1.37±0.57
TP+0.7HH+0.3P	11.48±0.20 *	14.67±0.42 *	-1.32±0.77 *	1.90±1.08 *
Ganoderma lucidum				
GL+ HH	13.23±0.39 *	14.63±0.19 *	3.22±1.26	3.01±0.95
GL+ 0.7HH+0.3P	14.69±0.08 *	16.72±0.73 *	2.46±0.69	3.24±1.02
GL+ RH	13.51±0.41 *	19.04±0.80 *	0.27±1.21	-0.24±0.48

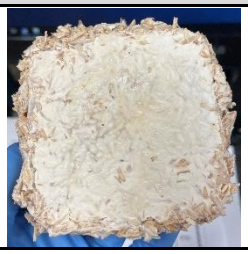
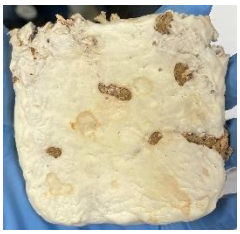
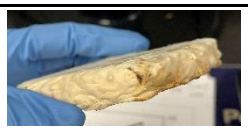
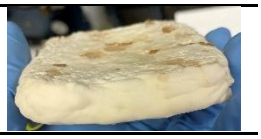
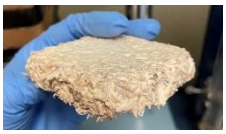
4.3.5 Mechanical Properties

4.3.5.1 Samples' appearance after compression

Table 4.6 illustrates the appearance of samples with and without compaction after compressive testing. All of the samples remained intact after compression. Since compacted samples had more mycelium skin on the surface, fewer materials were squeezed out of the sides than those without compacted. From the visual appearance, for samples TP+HH, TP+0.7HH+0.3P, GL+HH, GL+0.7HH+0.3P, and GL+RH, compaction appeared to reduce the samples' deformation after compressive tests.

Table 4.6 Top view and flank view of samples after compressing testing

Uncompacted Samples	TP+HH	TP+0.7HH+0.3P	FF+HH	FF+0.7HH+0.3P

Top view				
Flank view				
Compacted Samples	TP+HH	TP+0.7HH+0.3P	FF+HH	FF+0.7HH+0.3P
Top view				
Flank view				
Uncompacted Samples	GL+HH	GL+0.7HH+0.3P	GL+RH	
Top view				
Flank view				
Compacted Samples	GL+HH	GL+0.7HH+0.3P	GL+RH	
Top view				
Flank view				

4.3.5.2 Compressive strength and Young's Modulus

Appendix 5 shows the original stress-strain curves of all samples. Compressive strength and Young's modulus reflect the ability of MBMs to withstand compression and resist deformation when used as protective packaging.

Except for the compressive strength value at 10% strain for FF+0.7HH+0.3P, there are statistically significant differences in compressive properties between uncompacted and compacted samples.

For the compacted samples, compressive strength at 10% strain ranged from 0.018-0.155MPa (Table 4.8), showing competitive values compared to EPS, whose value ranges from 0.040-0.345 MPa (ASTM International, 2021). Compressive strength at 35% strain ranged from 0.097-0.653 MPa (Table 4.8), which is higher than the results of one previous study that ranged from 0.017-0.300 MPa (Tacer-Caba et al., 2020). It can be seen that compressive strength was higher in compacted samples than those without compaction (Table 4.7). For the compacted samples, there was an increase in compressive strength of 28.57-129.63% at 10% strain and 37.32-139.42% at 35% strain, respectively (Table 4.9).

Table 4.7 Overview of compressive strength (±SEM) and Young's modulus (±SEM) of uncompacted samples.

Materials	Compressive strength at 10% strain (MPa)	Compressive strength at 35% strain (MPa)	Young's modulus (Mpa)
FF+HH	0.027±0.002	0.113±0.006	0.262±0.015
FF+0.7HH+0.3P	0.028±0.001	0.142±0.002	0.282±0.009
TP+HH	0.027±0.001	0.104±0.002	0.255±0.005
TP+0.7HH+0.3P	0.033±0.004	0.136±0.013	0.324±0.033
GL+ HH	0.072±0.003	0.281±0.002	0.687±0.024
GL+ 0.7HH+0.3P	0.083±0.010	0.320±0.034	0.770±0.094
GL+RH	0.011±0.000	0.044±0.003	0.120±0.005

Table 4.8 Overview of compressive strength (±SEM) and Young's modulus (±SEM) of compacted samples

Materials	Compressive strength at 10% strain (MPa)	Compressive strength at 35% strain (MPa)	Young's modulus (Mpa)

FF+HH	0.048±0.002	0.208±0.022	0.467±0.032
FF+0.7HH+0.3P	0.036±0.003	0.195±0.013	0.360±0.028
TP+HH	0.062±0.003	0.249±0.005	0.618±0.013
TP+0.7HH+0.3P	0.050±0.001	0.195±0.001	0.501±0.021
GL+ HH	0.155±0.009	0.653±0.022	1.562±0.059
GL+ 0.7HH+0.3P	0.124±0.003	0.478±0.005	1.174±0.050
GL+RH	0.018±0.001	0.097±0.009	0.179±0.013

Table 4.9 The increase of dry density, compressive strength, and Young's modulus after compaction

Materials	The increase of compressive strength at 10% strain (%)	The increase of compressive strength at 35% strain (%)	The increase of Young's modulus (%)	The increase of dry density (%)
FF+HH	77.78	84.07	78.24	21.92
FF+0.7HH+0.3P	28.57	37.32	27.66	24.74
TP+HH	129.63	139.42	142.35	34.29
TP+0.7HH+0.3P	51.52	43.38	54.63	9.57
GL+ HH	115.28	132.38	127.37	21.93
GL+ 0.7HH+0.3P	49.40	49.38	52.47	20.74
GL+RH	63.64	120.45	49.17	33.63

The compressive Young's modulus of compacted samples varied between 0.179 and 1.562 MPa, which was higher than that of samples without compaction (0.120-0.770MPa), with a 27.66-142.35% increase (Table 4.9). However, the value was significantly lower than that of EPS, which is 5-40 MPa (Matweb, n.d.).

Additionally, MBMs' dry density is generally related to their mechanical properties (Tacer-Caba et al., 2020). Figure 4.9 shows the correlation between compressive Young's modulus and the dry density of compacted and uncompacted samples. Although some previous studies showed that the dry densities of MBMs do not always positively correlate with Young's modulus (Girometta et al., 2019), for all the protocols in this study, compacted samples had higher density and compressive Young's modulus than those without compaction. This result demonstrated that compaction could simultaneously enhance MBMs' dry density and mechanical properties. When using compaction to improve MBMs' mechanical properties, the protocols that showed a higher increase in Young's modulus and a lower increase in density would be more meaningful. For example, comparing samples TP+HH and GL+RH, they had similar increases in density after compaction, but the

increase in Young's modulus of the former one almost doubled the latter. Furthermore, the effect of compaction on improving the mechanical properties depends on the samples' recipes. For instance, the increase in Young's modulus for samples made of pure hemp hurds almost doubled that for hybrid substrate-based samples, and samples produced from rice husks exhibited the lowest increases among all pure substrate-based samples.

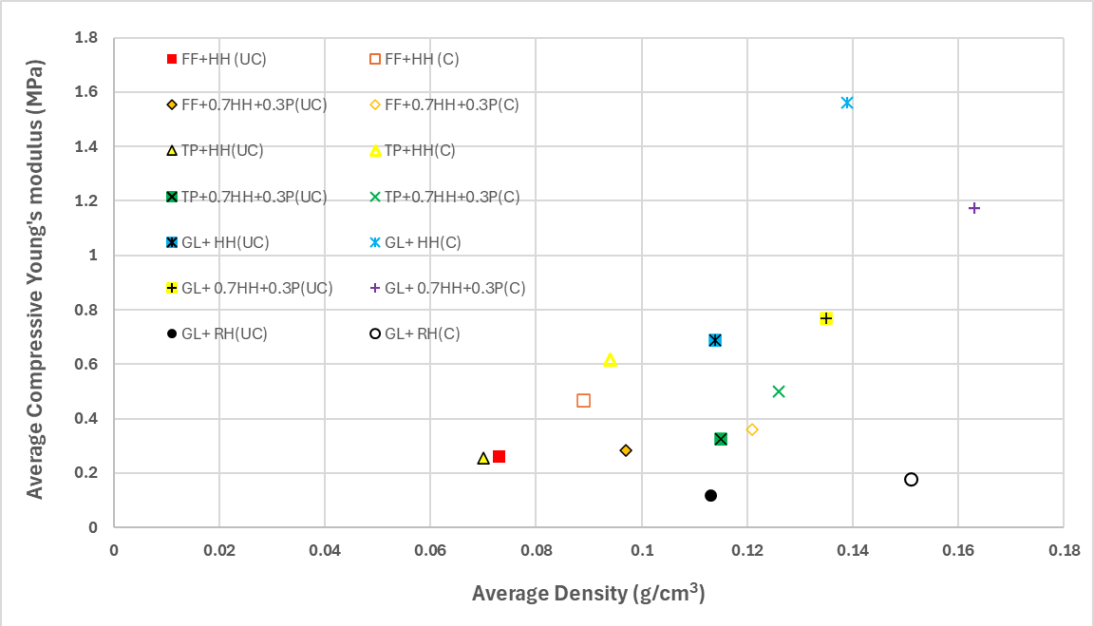


Figure 4.5 Comparison of dry density and compressive Young's modulus of samples. UC represents uncompacted samples, and C represents compacted ones.

4.4 Conclusion

This study evaluated the impacts of compaction on the properties of MBMs with different recipes. Morphology, dry density, water absorption, compressive strength, and compressive Young's modulus were assessed on MBMs with compaction and compared to those without compaction.

Visual and light microscope observations showed that compacted samples had similar or thicker mycelium skins outside than those without compaction. However, compacted samples GL+RH and TP+0.7HH+0.3P had significantly less mycelium inside than uncompacted ones. The lack of air inside compact samples and challenging substrates were accreted to this phenomenon.

Compacted samples in this study showed an increase of 9.57-34.29% in dry density. The dry density of compacted samples ranged from 0.089 to 0.163 g/cm^3, which was higher than that of EPS but lower than pulp molding packaging.

The overall weight gain of compacted samples after moisture exposure was 4.59-6.98% at 60% RH and 11.02-19.04% at 80% RH. The compaction method did not necessarily increase or decrease the water absorption of samples, depending on their recipes. In addition, the impact of compaction on the change in thickness of samples after moisture exposure was not significant for most samples in this study.

Compacted samples show improvement in compressive properties. Compressive strength increased 28.57-129.63% at 10% strain, 37.32-139.42% at 35% strain, and compressive Young's modulus increased 27.66-142.35%. It is worth noting that density increased along with enhanced mechanical properties.

Overall, this study showed that the compaction method had some benefits, like enhancing MBMs' compressive properties. However, it also brought some troubles, like increasing dry density and inhibiting mycelium growth for some samples. Furthermore, this study demonstrated that the extent of compaction impacts varied from recipe to recipe, especially for water absorption. In future studies, exploring the recipe that mycelium could grow well inside the compact samples would be valuable because the abundance of mycelium inside could reduce the dry density of MBM. In addition, comparing different thicknesses of MBMs to find a suitable size would be another way to solve the issue of mycelium thriving inside MBMs.

Chapter 5 Summary and Conclusions

This study evaluated the impacts of hybrid substrates and the compaction method on the properties of MBMs.

Two kinds of hybrid substrates were used for producing MBMs: 70% hemp hurds+30% waste paper, and 60% hemp hurds+40% waste paper. Only *Ganoderma lucidum* could grow on the mixture substrates with 40% paper successfully among the three fungal species used in the study. Morphology, dry density, water absorption, compressive strength, and Young's modulus were assessed on seven MBMs protocols. From the perspective of visual appearance, FF+0.7HH+0.3P had a velvety and foam-like appearance, GL+0.7HH+0.3P and GL+0.6HH+0.4P exhibited a more compact structure, and TP+0.7HH+0.3P showed a loose structure with less mycelium skin on the surface. Samples' dry densities increased along with the increase of the waste paper ratio in the substrates, indicating that mixing waste paper raises the density of MBMs. The dry density of samples fed with hybrid substrates also varied among different fungal species, with GL-based samples tending to have the highest density ($0.145 g/cm^3$) and FF-based samples having the lowest density ($0.097 g/cm^3$). This suggests that both paper ratio and fungal species impact MBMs' dry density, which is an essential property for packaging material. The final weight increase was higher in samples exposed to 80% RH than in those exposed to 60% RH, with hybrid substrate-based samples showing an 11.48-15.42% and a 2.93-5.68% final weight increase at 40 ℃, respectively. Samples made of hybrid substrates appeared to have better water resistance at 60% but worse at 80% RH than those made of pure hemp hurds, indicating the limitation of application conditions of MBMs with hybrid substrates. Compressive properties analysis revealed that samples made with hybrid substrates had higher values than those made with pure hemp hurds. This suggests that incorporating waste paper into the substrates can improve MBMs' compressive properties. However, excessive paper added to the substrates would lead to the decline of compressive properties.

The effects of compaction on the properties of MBMs with different protocols were also evaluated. Morphology, dry density, water absorption, compressive strength, and compressive Young's modulus were assessed on MBMs with compaction and compared to those without compaction. Visual and light microscope observations showed that compacted samples had similar or thicker mycelium skins outside than those without compaction. However, compacted samples GL+RH-

55 and TP+0.7HH+0.3P-55 had significantly less mycelium inside than uncompacted ones, and GL+RH samples had the least mycelium inside. The lack of air inside compact samples, plus the difficult-to-digest substances, may make it difficult for hyphae inside these samples to grow. Compacted samples in this study showed an increase of 9.57-34.29% in dry density. The dry density of compacted samples ranged from 0.089 to 0.163 g/cm^3, higher than that of EPS but lower than pulp molding packaging. The overall weight gain of compacted samples after moisture exposure was 4.59-6.98% at 60% RH and 11.02-19.04% at 80% RH. However, the compaction method did not necessarily increase or decrease the water absorption of samples, depending on their recipes. In addition, the impact of compaction on the change in thickness of samples after moisture exposure was not significant in this study. The improvement of compressive properties can be seen in compacted samples. There was an increase in compressive strength of 28.57-129.63% at 10% strain, 37.32-139.42% at 35% strain, and a 27.66-142.35% increase in compressive Young's modulus. It is worth noting that the density increased along with enhanced mechanical properties.

In conclusion, this study upcycled waste paper to create MBMs, which showed enhancements in physical and mechanical properties compared to those made of pure hemp hurds. These improvements included a foam-like appearance, acceptable dry density, higher compressive properties, and better water resistance at 60% RH. Additionally, the compaction method showed some benefits, like enhancing MBMs' compressive properties, but it also brought some troubles, like increasing dry density and inhibiting mycelium growth for some samples.

In future studies, it would be valuable to investigate the mechanism behind the increased water absorption of MBMs fed with waste paper-based substrates at higher relative humidity and determine the optimal waste paper ratio in substrates to improve their properties. Furthermore, screening mycelium species capable of colonizing waste paper may further enhance MBMs' properties, and exploring other low-value materials, such as tea waste and used coffee grounds, as potential substrates may reduce manufacturing costs. Finally, it is worth exploring the fabrication methods and recipes that help mycelium grow well inside the compact samples because the abundance of mycelium inside compacted composites could reduce MBMs' dry density while improving the compressive properties of MBMs.

Chapter 6 Engineering Significance

This research shows that MBMs with hybrid substrates or compaction have the potential to be used as environmentally friendly packaging materials in terms of dry density. The lower dry density of MBMs produced in this study can reduce the shipping costs compared to the currently widely used eco-friendly packaging materials.

The feasibility of incorporating waste paper into the substrates partially addressed the industries' concerns about substrate costs and availability. This indicates that more low-value materials should be investigated to produce MBMs in the future.

The compaction method could be taken into account in the large-scale production. However, mechanical pressurization should be utilized instead of hand compaction, improving work efficiency and standardization. Furthermore, MBMs with different compaction levels could be produced using mechanical pressurization, catering to various applications.

Although incorporating waste paper in the substrates and compaction increased MBMs' dry density, it also enhanced their compressive properties. Therefore, adding waste paper to the substrates or the compaction method could be considered for some applications, such as construction materials, where the compressive properties are essential and the dry density is unimportant.

Overall, this research provides methods to reduce the substrates' costs and improve MBMs' properties. The findings also offer the possibility of various applications except for packaging materials.

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Appendix

1. Mycelium-based materials without compaction before dehydration

FF+HH

FF+0.7HH+0.3P

TP+HH

TP+0.7HH+0.3P

GL+ HH

GL+ 0.7HH+0.3P

GL+ 0.6HH+0.4P

GL+RH

2. Mycelium-based materials without compaction after dehydration

FF+HH

FF+0.7HH+0.3P

TP+HH

TP+0.7HH+0.3P

GL+ HH

GL+ 0.7HH+0.3P

GL+ 0.6HH+0.4P

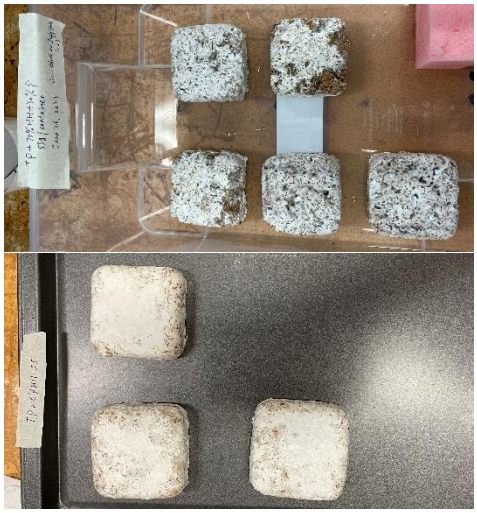
GL+RH

3. Mycelium-based materials with compaction before dehydration

FF+HH

FF+0.7HH+0.3P

TP+HH



TP+0.7HH+0.3P

GL+ HH

GL+ 0.7HH+0.3P

GL+RH

4. Mycelium-based materials with compaction after dehydration

FF+HH

FF+0.7HH+0.3P

TP+HH

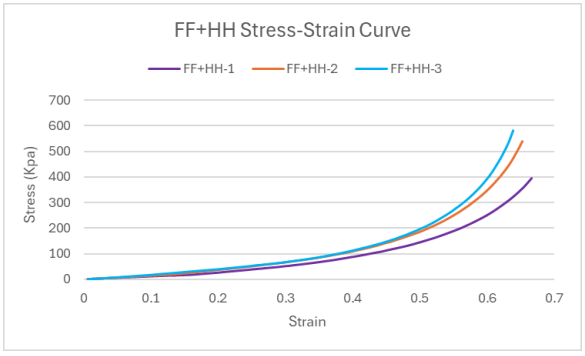
TP+0.7HH+0.3P

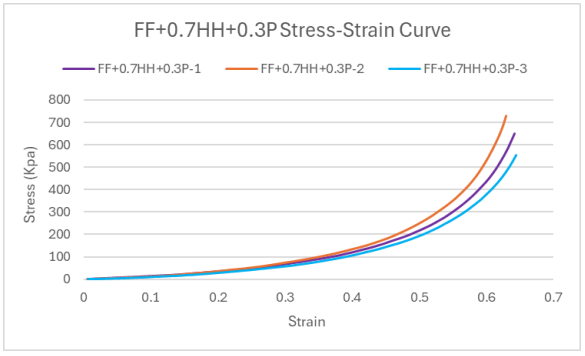
GL+ HH

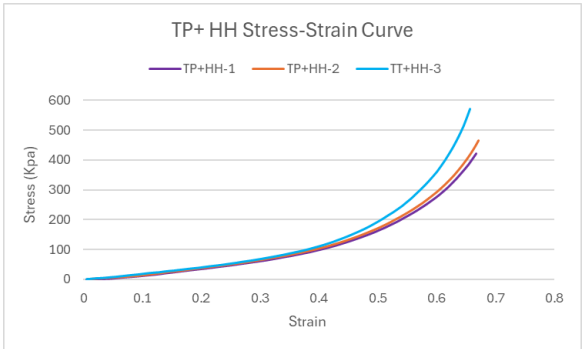
GL+ 0.7HH+0.3P

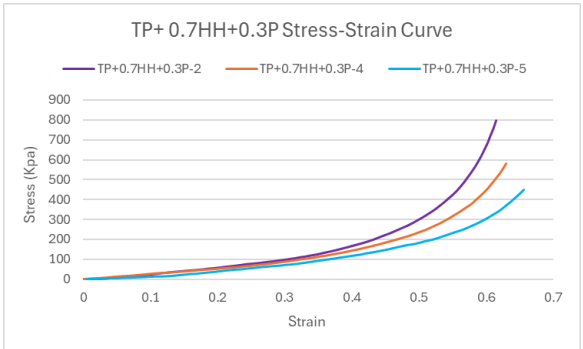
GL+RH

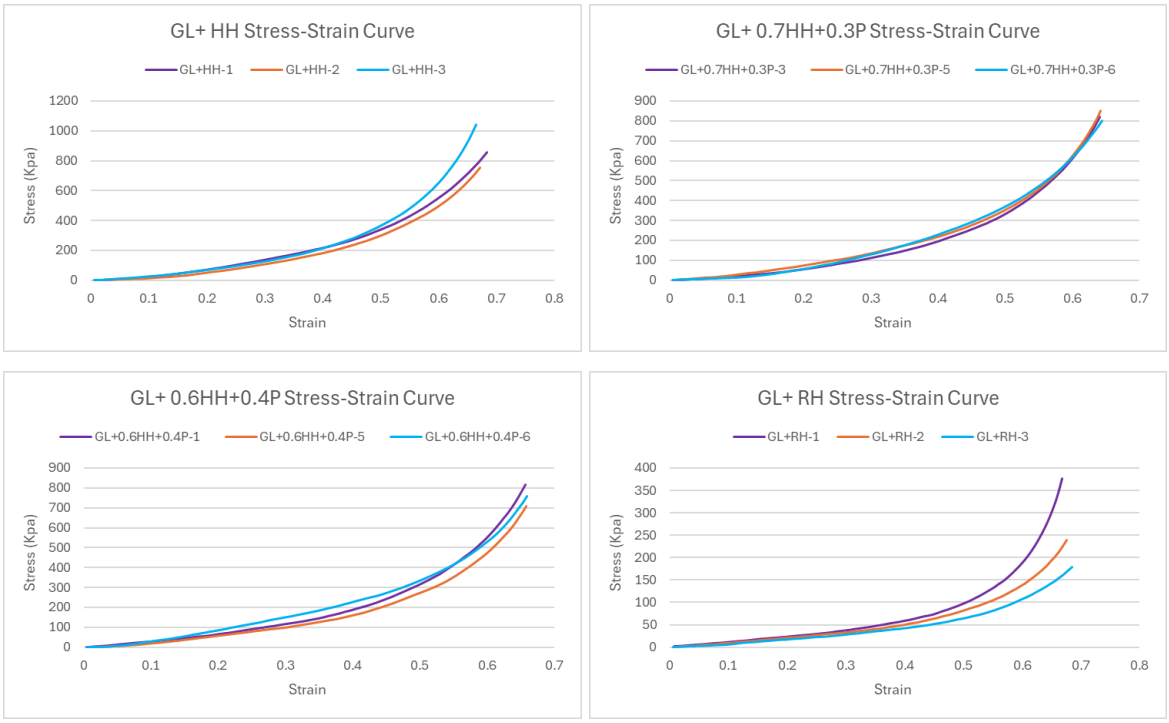
5. Original Stress and strain curves for mycelium-based materials without compaction



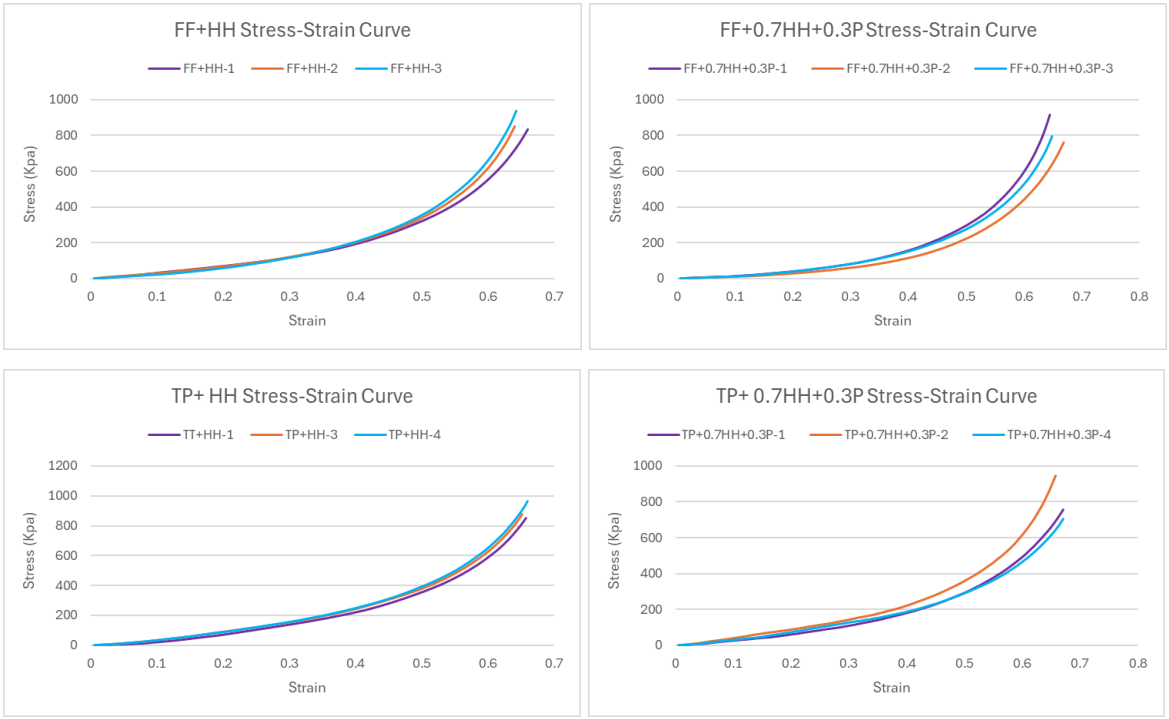


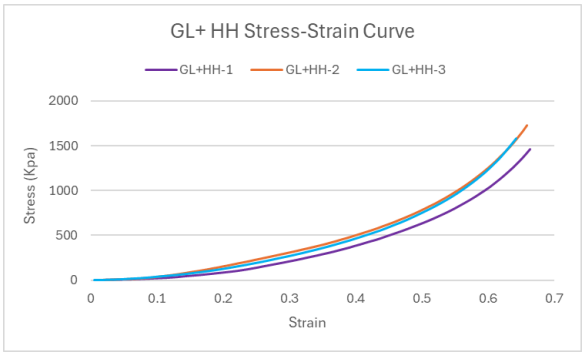


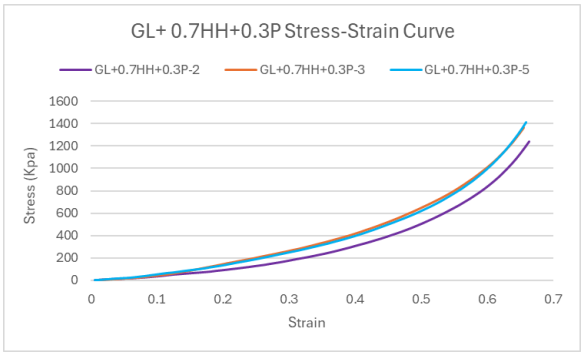


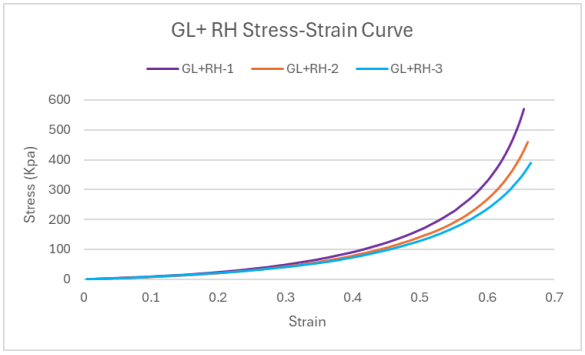


6. Original Stress and strain curves for mycelium-based materials with compaction




GL+ HH Stress-Strain Curve
GL+HH-1
GL+HH-2
GL+HH-3
Stress (Kpa)
2000
1500
1000
500
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Strain


GL+ 0.7HH+0.3P Stress-Strain Curve
GL+0.7HH+0.3P-2
GL+0.7HH+0.3P-3
GL+0.7HH+0.3P-5
Stress (Kpa)
1600
1400
1200
1000
800
600
400
200
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Strain


GL+ RH Stress-Strain Curve
GL+RH-1
GL+RH-2
GL+RH-3
Stress (Kpa)
600
500
400
300
200
100
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Strain