

The Impact of Magnesium Sulfate Supplementation and Growth Duration on the Properties of Mycelium-based Composites and Chitin Expression

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Abstract

Mycelium-based composites (MBCs) are eco-friendly renewable alternatives to fossil-fuel based foams and composites, bringing forward resource efficiency, biodegradability, carbon sequestration, and green bioengineering. Produced from agricultural waste and fungal mycelium, the physical and mechanical properties of MBCs vary depending on the growth conditions of material production, after the choice of the substrate and fungal species. The tunability of the key properties of hemp-hurds based MBCs was investigated by varying growth time and supplementation with MgSO_4 using *Ganoderma lucidum* and *Trametes pubescens*. The properties of the MBCs were investigated through unconfined compression force deflection, Fourier transform infrared (FTIR) spectroscopy, transient thermal conductivity measurement, and various measurements of physical characteristics. Compressive strength at 10% strain ranged between 43.11-73.23 kPa, with the highest strength belonging to the *G. lucidum* magnesium supplemented short-cultivation treatment. The thermal conductivity of the samples differed minimally within the 0.0495 to 0.055 $\text{W}\cdot\text{m}^{-1}\cdot\text{K}^{-1}$ range. FTIR analysis suggested that MgSO_4 supplementation may increase mycelial chitin content in a species-dependent manner. Overall, *T. pubescens* produced composites with lower density and Young's modulus but higher water absorption rate (WAR), characteristics that may be advantageous for certain packaging applications. *G. lucidum* delivers a biocomposite with higher compressive strength and low WAR, ideal for applications in the construction industry.

Acknowledgements

Education, at its core, is a practice of growth. It is the gradual expansion of understanding, the refinement of thought, and the cultivation of the capacity to question, to connect, and to transform. It is, fundamentally, a human process, one that extends beyond institutions and persists irrespective of them.

Academia, however, operates as a system. It organizes knowledge, structures inquiry, and defines legitimacy. In doing so, it does not merely facilitate education; it shapes the conditions under which knowledge itself becomes recognizable. In this sense, and following the work of Michel Foucault, knowledge is not independent of power but is constituted through it. What is accepted as truth, what is funded, and what is ultimately pursued emerge from institutional arrangements that both enable and constrain inquiry.

Within this framework, research may be directed not solely by urgency or necessity, but by visibility, established pathways, and interpretations of broader priorities. These patterns reflect what Foucault describes as regimes of truth: systems through which certain forms of knowledge are validated and reproduced while others remain marginal. The academic subject, too, is shaped through evaluation, publication, and peer recognition, which establish norms of productivity, methodology, and legitimate inquiry. This is not necessarily a failure, rather, an indication that academia functions according to its own internal logic that does not always align with the broader aims of education as a transformative and ethical practice.

Ethics occupy an ambiguous position within this structure. While articulated as essential, they are frequently operationalized as procedural requirements rather than principles that actively govern conduct. Such dynamics are inseparable from historical context; institutions carry the imprints of their formation, and contemporary practices often reflect inherited structures of authority and knowledge. To recognize this is to acknowledge that systems of knowledge production evolve within histories that continue to shape their present configurations.

And yet, within and despite these structures, education persists.

It persists when the desire to grow meets the desire to nurture growth. It persists in moments of genuine inquiry, in conversations that extend beyond formal requirements, and in the individual effort to understand rather than merely to complete. It exists wherever learning is pursued as an end in itself, rather than as a metric to be satisfied.

For that, there is gratitude. Gratitude to those individuals who, within the constraints of the system, continue to create space for inquiry that exceeds what is prescribed. Gratitude for moments in which learning resists reduction to output, and for the persistence required to sustain it.

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Contribution of Authors

This thesis is organized as a "sandwich" style or grouped-manuscript thesis with chapter 2 containing material from journal articles pending publication submission. The following describes the title of the manuscript and the contribution of authors to this work:

- Chapter 2 includes content from a research manuscript titled “The Impact of Magnesium Sulfate Supplementation and Growth Duration on the Properties of Mycelium-based Composites and Chitin Expression”.

Author contributions:

Ava Tavakoli (Thesis author): Conceptualization, Visualization, Methodology, Experimentation, Software, Analysis, Writing of original manuscript

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Chapter 1: State of the Art of Mycelium-based Composites

Introduction and Objectives

The negative impacts of the linear extract-produce-use-dump economic model have emphasized the need for transition towards sustainable practices. The alternative to the linear economy has been described as the Circular Economy (CE), in which materials are recovered to be reused or repaired first, remanufactured second, and only after considered for traditional recycling which involves raw material utilization [1]. Through its environmental, economic, and social dimensions, the CE can result in resource security, environmental impact mitigation, and regulatory alignment. Both replacement of fossil fuel-based products and valorization of waste streams fall within the scope of sustainable practices that direct CE development, brought forward by Mycelium-based Composites (MBCs).

Expanded polystyrene (EPS) – commercially known as Styrofoam - is a widely used petrochemical derivative with a manufacturing process that has high global warming potential [2]. EPS is not biodegradable and disperses easily, and due to its lightweight nature, its recycling is not considered feasible in many regions, leading to much EPS in the landfills. There, it contributes to further greenhouse gas emissions and may leach styrene monomers into surrounding land and water and harm wildlife, resulting in its high footprint [2, 3]. Hence, alternatives to EPS that mitigate its negative impacts - such as mycelium-based composites (MBCs) - become strategic pursuits towards a CE. Previous reports indicated that minimally processed hemp mycelium composites can meet or exceed EPS compressive strength standards [2]. Although density and water absorption parameters remain different between EPS and MBCs, potential for substitution in specific applications persists. Assessments revealed up to 89% lower energy demand and 72% lower CO₂ emissions for MBCs compared to EPS, because of the lower global warming potential of MBCs [4].

Wood-based composites are similarly problematic in that their manufacturing involves using petroleum-derived binders such as urea formaldehyde and phenolic resins under high pressure and temperature [5]. The manufacturing of these adhesives both accelerates resource depletion and causes the release of volatile organic compounds, threatening environmental and

human health. Natural alternatives such as starch and soy protein have been produced, but introduce chemical residues and have a costly purification process [5]. MBCs hold comparable mechanical properties to wood-based composites, particularly in applications for acoustic and thermal insulation or ceiling panels.

Agricultural residues are also burdensome when not valorized, as they are either burnt or landfilled, causing environmental pollution and greenhouse gas emissions [4]. Diverting this resource to produce valuable materials will contribute to moving towards a CE, while also replacing undesired disposal pathways. In MBCs, the fungus binds the particles of a substrate that is often sourced through an agricultural waste stream, resulting in a low-energy low-carbon, biodegradable material. Some studies consider MBCs as carbon sinks or climate-negative materials because the plant-based substrate captures CO₂ from the atmosphere during its growth, resulting in the material sequestering more carbon than it emits [6].

MBCs are porous biocomposites in which the fungal mycelium acts as an adhesive, binding the lignocellulosic biomass particles and forming a chitinous network through its growth that supports the biocomposite structure. Performance of MBCs strongly depends on substrate characteristics, species of choice, and fabrication conditions. Reported practical applications of MBCs are in packaging, thermal and acoustic insulation, interior architecture, lightweight panels, and other low load applications [7, 8]. The environmental logic in support of MBCs is systemic where they can divert biomass from burning or dumping and replace fossil-derived materials and binders, while delivering comparable performance. The properties of MBCs remain inferior to competing alternatives thus far, and several limitations such as high hygroscopicity and low tensile strength need to be addressed [9]. Therefore, expanded investigations on pathways for achieving controlled material properties remain essential.

The present work aims to contribute to the elucidation of the effect of cultivation parameters on the properties of MBCs. A quantitative understanding of the impact of three biological parameters – namely species, growth duration, and MgSO₄ supplementation - on the mechanical properties and chemical composition of MBCs has been pursued. The objective of the investigation is to illuminate the best setting for the three parameters in various application end-uses. The state of the art of MBC production has been thoroughly reviewed (in the first chapter) to aid in the design of an empirical study (in the second chapter) that evaluated the tunability of MBC

physical, mechanical, and chemical properties by varying growth duration and providing magnesium sulfate supplementation.

State of The Art of Mycelium-based Composites

The state of the art has shifted from proving that MBCs can work to engineering them with predictable performance, compliance with standards, and scalability. The literature consistently reports low thermal conductivity and strong sound absorption along with good mechanical strength, well-establishing the use-case of MBCs. However, limitations such as variability in properties, moisture sensitivity, and durability persist [10, 11]. The leading edge is interface engineering, genetic or biochemical programming, additive manufacturing, predictive modeling, and standardized testing [12].

Three clear frontiers can be identified in the state of the art of MBCs. The first involves the persistence of biology as an engineering lever, where inherent mycelial properties such fungal-wall chemistry or mycelial density control the mycelium's physical performance in MBCs. Efforts in this direction began with examples such as the work of Appels et al. in 2018 where it was demonstrated that hydrophobin gene deletion in *Schizophyllum commune* MBC yielded 3-4 times higher Young's modulus and tensile strength [13]. Work in this field continues to 2026 examples such as the successful breeding of *Pleurotus ostreatus* for the disruption of the *mbpl* gene (a transcription factor affecting normal mycelial growth and cell wall synthesis), which resulted in higher Young's moduli and ultimate tensile strength [14]. Regardless, public perception and adoption of genetically modified organisms remain challenging. Second, the field is advancing towards predictive material design to complement experimental material design. Examples include the use of artificial neural networks in predicting the mechanical performance of MBCs, with high coefficients of determination that capture the majority of experimental variability [15]. Third, modifications through interface and surface engineering are explored for enhancing performance in various applications, such as polydopamine-modified MBCs with heightened bending strength and internal bonding strength [5].

Techno-Economic Analysis

Techno-economic analysis (TEA) is an evaluation of the technical and economic aspects of a process or a projection of service performance that discusses the process costs and benefits to enable decision-making for investments. The literature on MBC TEAs lacks industrial projects and

remains limited to review-derived work, LCAs that indirectly discuss cost, or lab-scale substitutes. Generally, MBC production costs vary relative to conventional materials, influenced by specific application, raw material availability, production approach and scale, labor costs, and the market. It has been reported that MBC cost savings compared to paper-based materials exceed 65% and reach over 90% relative to fabric composites, gypsum-based materials, polymer materials, and wood-PHA composites [16].

TEA examples on MBCs include the work of Jiang et al. in 2016, on the application of MBCs in biocomposite sandwich structures where manufacturing parameters were measured for each step to calculate the equivalent uniform annual costs (UAC) of manufacturing facilities. This was followed by the assembly of a manufacturing line cost model that determined the optimal number of machines and workers, to use for calculating the net cost of each part and retail price. In this case, optimization reduced unit cost from \$53.06 to \$49.15, emphasizing that while process planning may move the economics, the baseline system is still relatively labor-intensive and costly [17]. Comparison of cost per unit of weight estimates the cost of MBCs at \$0.12/kg, while paper-based materials, polymer materials, and wood-PHA composites cost \$0.35/kg, \$2.2/kg, and \$3.35/kg, respectively [16]. In another study, MBCs produced from agricultural and food waste were evaluated at a laboratory scale to cost €0.3 to €0.6/kg (approximately \$0.34 to \$0.69 USD/kg), while the cost of EPS is about €1.68/kg (~\$1.92/kg) in Europe, demonstrating the economic competitiveness of MBCs [4]. Although cost comparison is difficult due to the dependency of cost on various factors such as region and process, MBC competitiveness is possible through the careful selection of process parameters.

Previous reports indicate labor as a primary driver of cost and suggest automation as the solution [18]. Energy usage in electricity for sterilization, incubation, drying, and climate control is the second major driver, and it was found to be the largest contributor to most LCA impact categories [19]. The economics of raw substrates stand out after labor and energy. While lignocellulosic raw material may advantageously be cheap or free to source and collect, preprocessing, sterilization, or drying energy consumption add to the costs [20]. Generally, economics improve through process simplifications such as shorter growth cycles, minimal substrate pretreatment, reuse of molds, as well as automation.

Life Cycle Assessment

Life cycle assessment (LCA), which is a systematic quantification of the environmental impacts of products, has been well studied for MBCs. Applications of MBCs studied for their life cycle include packaging, bricks, and thermal and acoustic insulation [19, 21, 22, 23]. MBCs as packaging box inserts had a lower impact than EPS inserts in terms of carbon footprint and energy, and a higher impact in stratospheric ozone depletion and marine eutrophication [22]. Another study investigated the application of MBCs as insulation and observed encouraging results when considering climate change, but noted trade-offs where MBCs performed worse in other categories [21].

Energy consumption dominates most results. It has been reported that energy use for drying and incubation during production of acoustic insulation panels has the greatest contribution to most impact categories, including global warming potential [19]. In the life cycle of MBCs as bricks, 81.4% of total CO₂ eq. for climate change was from electricity for sterilization, incubation, and drying [22]. Generally, manufacturing and the associated electricity use dominate life cycle impacts, and the release of stored biogenic carbon begins during manufacturing [19]. Alaux et al. assessed a cradle-to-grave model in which electricity was further confirmed as the main residual green-house gas (GHG) driver [24]. The substantial influence of electricity on the LCA was verified across geographies where the assessment of the impacts of the technology in Africa likewise assign the pre-processing and post-processing energy demand as major drivers [23].

The effect of substrate is also significant when evaluating the environmental impacts of MBCs. For example, hemp shives and grain mix contributed 77.7% to eutrophication and 81.8% to land use [22]. Substrate choice is a key factor in determining environmental performance, according to a study that compared MBCs of *Lentinus tigrinus* with different substrates. Ash-wood chips held the lowest GWP at -9.77 kg CO₂ eq, while straw-based MBCs held the highest GWP at 4.04 kg CO₂ eq [25].

The carbon performance of MBCs is often good, negative in some cases, yet not always comparable to conventional alternatives. Livne et al. reported 860 MJ.m⁻³ embodied energy, which was about 1.5 to 6-fold less than common construction materials. They further reported -39.5 kg CO₂ eq.m⁻³ embodied carbon, of which 73% belonged to manufacturing and 21% to metabolic emissions [26]. Volk et al. reported 0.3668 kg CO₂ eq.kg⁻¹ of material under lab scale production for

hemp-based MBC, which was a superior performance relative to extruded polystyrene, quadcore sandwich panel, foam concrete, and rockwool [27]. The climate change impact of MBC bricks was reported at 0.494 kg CO₂ eq. [22]. The global warming potential (GWP) of the application of MBCs as packaging corners for electronics materials was found to be either 1.23, 2.16, or 3.24 kg CO₂ eq., compared to EPS at 3.35 kg CO₂ eq. [28]. Furthermore, the application of MBCs in timber frame construction demonstrated enhanced carbon performance relative to conventional sandwich panels [6]. Evaluations of the effect of scale-up reveal that while it can improve the environmental case of MBCs, it does not erase trade-offs. The work of Alaux et al. expands assumptions to industrial scale, concluding that while most impacts improve with scale-up, terrestrial ecotoxicity and eutrophication get worse [24].

Production of Mycelium-based Composites

Since the properties of MBCs can be modified through the careful adjustment of the manufacturing bioprocess, much attention in the literature has been directed to studying the effect of various bioprocess parameters. Despite this effort, production is not standardized, and manufacturing strategy varies across different studies, yielding different results. Various aspects of the production of MBCs have been reviewed across the literature for their effect on properties of MBCs and have been reviewed as follows.

Substrate Selection

The substrate's physical and chemical nature contribute significantly to the final properties of the MBC and are main control variables in MBC manufacturing. The substrate affects growth, colonization, density, strength, hydrodynamic behavior, and even environmental impacts. The primary requirement for a substrate is chemical suitability and compatibility with the selected species. A suitable substrate provides enough cellulose and possesses the right pH, organic carbon content, total nitrogen, and optimal C:N ratio [16].

The physical form of the substrate has a critical impact on the MBC. A study comparing sawdust as substrate with shredded beech wood, oak shavings, tree of heaven chips, and wheat straw demonstrated that despite the choice of the species, saw dust produced a brittle material that crumbled easily [29]. This characteristic was attributed to its fine physical form that did not provide enough structure to the composite nor adequate surface area for the fungus to bind the particles together. It has been reported that the size of substrate fibers impacts mechanical

properties of MBCs more than its chemical composition [30]. Particle size has been emphasized as key since it brings about a balance between surface availability and airflow. It must also be carefully selected as a design parameter: finer for denser homogeneous MBC with high compressive strength and coarse for lightweight porous MBCs [20].

Economic factors for a circular economy are also notable in substrate selection. The local availability of a substrate avoids transportation-associated emissions. Diversion of local waste streams is beneficial on environmental grounds by delaying waste management. A universally ideal substrate has not been named, and the choice of the substrate is specific to application and geography.

Species Selection

The selection of the species is a primary design factor that affects the type and extent of binding and the performance levels that the material can reach. The biological differences between species such as varying growth rates, enzymatic profiles that yield varying lignin and cellulose degradation degrees, and mycelium filament thickness and morphologies generate distinct structural architectures that place importance on the role of species [31]. The impact of species selection on tensile strength, compressive strength, and density has been documented, and the success of the bioprocess depends on intentional species selection [31, 32].

Depending on the lignocellulosic degradation effect that species demonstrate, fungi can be categorized into soft-rot, white-rot, and brown-rot, among which white-rot Basidiomycetes dominate MBC production literature [9, 33]. White-rot degradation has been given preference as it avoids the defibration of wood during degradation, even during advanced processes, and minimizes volumetric shrinkage-associated changes in manufacturing. Brown rot and soft rot fungi are used less frequently as they consume holocellulose, which undermines material strength [9]. Among white-rot Basidiomycetes, three genera of *Pleurotus*, *Ganoderma*, and *Trametes* are the most cited [32]. Reports on usage percentage of species across the field position *Pleurotus ostreatus* at 28%, second to *Ganoderma lucidum* at 44% [7].

The root-like structure of higher fungi, the mycelium, is composed of a network of chitinous tubes named hyphae, that expand through the substrate and absorb nutrients as they decompose the lignocellulosic substrate material through enzymatic degradation. Most of the composition of the mycelium includes glucans, chitin, proteins, tannin, and lipids [33]. With the ability to grow on

its own without cellular activation for forming fruiting bodies, the mycelium has been found to be one of the largest single organisms in some forests, encompassing kilometers of forest area underground. At the hyphal tip, a range of biochemical processes enable the degradation of surrounding biomass to facilitate hyphal elongation [34]. Hyphae are formed from a stream of cells that may or may not be separate from each other with a septate. The driving forces behind hyphal extension are the mechanisms regulating turgor pressure at the hyphal tip. The viscoelastic properties of the cell wall at the hyphal tip are partially attributed to the heightened activity of chitinase in this region. During hyphal extension, new membrane and wall material are inserted to the tip concurrent to the delivery of new cytoplasmic organelles to the apex, supported by the consistent production of vesicles behind the tip [34].

The mycelium is comprised of cell-like compartments housed within tubular hyphae, and cytoplasmic components can be exchanged among these compartments through microscopic openings in the cross-walls (septa). The cell-like components of the mycelium are contained within a protective tubular cell wall that delivers the mechanical properties of the mycelium. The cell wall of filamentous basidiomycetes is comprised of (1-3)/(1-6) β -glucan-chitin complex, (1-3) α -glucan, and glyco-proteins in general [35]. The cell wall components are added to the cell wall independently at the hyphal apex. The glucan-chitin complex components are synthesized as water soluble (1-3) β -glucan and chitin, with an initial hydrated gel-like structure in the cell wall before becoming cross-linked to chitin and introducing (1-6) β linkages [35]. The new bonds result in an alkali-insoluble β -glucan-chitin complex that changes the mechanical properties of the wall. The cell wall therefore holds a highly plastic texture at the hyphal tip with increasing firmness towards subapical regions. Apart from this insoluble complex, many filamentous fungi produce mucilage or slime, comprised of water-soluble (1-3) β -glucan with single (1-6) β -linked glucose [35]. The proteins of the outer layer of the cell wall include mannoproteins and hydrophobins [33]. Hydrophobins are a group of surface proteins and considered a component of the cell wall that are exclusive to fungi and essential for the formation of areal hyphae and adhesion to surfaces [33]. As surface-active amphipathic proteins, hydrophobins possess distinct hydrophobic and hydrophilic surface regions. The self-assembly of the amphipathic monolayer of hydrophobins on fungal interfaces results in an arrangement of the protein with the hydrophobic region facing outward, contributing to the highly hydrophobic fungal surface [36].

The hyphal network of the mycelium can be categorized as generative, skeletal, or binding hypha [33]. Generative hypha can form reproductive structures and generally hold thinner walls with many septa and clamp connections. Skeletal hypha are rarely branched, hold a limited number of septa, and are of longer and thicker structure. Binding hypha have rigid walls and are often branched [33]. Depending on the hyphal type available in a mycelial network, the mycelium can be categorized as monomitic, dimitic, and trimitic. Monomitic hyphal networks are comprised of only generative hypha, and dimitic and trimitic hypha are comprised of two or three types of hyphal network, respectively [33]. Trimitic systems experience a branched network with good adhesion. This categorization plays a definitive role in the performance of a specific fungus in MBCs, as reports indicate that more hyphal types result in better performance [9].

A list of eleven criteria describing ideal characteristics of a fungus for MBC production proposed by Sydor et al. has emerged as a framework for species selection: (1) rapid growth, (2) high virulence, (3) dimitic or trimitic hyphal structure, (4) white rot fungi, (5) high versatility in nutrition, (6) tolerance to a wide range of substrates and environmental conditions, (7) susceptible to readily controlled ecological factor, (8) easy to deactivate, (9) saprophytic, (10) non-mycotoxic, and (11) capable of producing natural active substances [9].

Substrate Pre-processing

The substrate used in MBC production serves as both a structural foundation and a nutritional source for the fungus, therefore its preparation directly impacts mycelial colonization, mechanical strength, water absorption, and biodegradability. Controlled substrate pre-processing can both enhance the bioactivity of the growth medium and improve interfacial adhesion, porosity, and network continuity [20].

The lignocellulosic substrate used in the production of MBCs is often pre-treated for modification in various ways, categorized into physical, chemical, and biological treatments [18, 20]. In physical treatments, such as the corona treatment or plasma treatment, the chemical composition remains intact, while the structural features are altered [18]. Broadly, physical treatment can be categorized into comminution, thermal treatments such as autoclaving, pasteurization, drying, mechanical densification, and sieving [20]. In a study using *Trametes versicolor* and five different substrate types, the mechanical performance of the MBCs held a

stronger correlation with the fiber processing (loose, chopped, pre-compressed, or tow) and particle size rather than the chemical composition of the fiber [37].

Chemical treatments on the other hand, which change the chemical composition of the substrate, include saline, alkaline and acid hydrolysis, acetylation, maleate coupling, oxidative treatments, and photocatalysis [20, 38]. Lastly, biological treatment can include enzymatic pre-digestion of the substrate or microbial co-culturing [20].

Spawn Cultivation

The spawn acts as the seed in mycelium production, where the mycelium is grown on a carrier material prior to introduction to the substrate. Production methods excluding the spawn growth stage have been reported and produce a product with less desirable mechanical properties. Sun et al. compared the introduction of pure mycelium particles in MBC production to introduction as wood-mycelium particles produced through the solid state fermentation (SSF) of wood particles. It was reported that the latter produced a product with relatively superior physical and mechanical properties [39].

Spawn can be produced through SSF, which involves the growth of a living organism on a solid substrate. Examples of SSF spawn include sawdust spawn and grain spawn, which have also been referred to as the mother culture in some bioprocesses. Grain spawn is often selected for its nutritional properties, and grains such as rye, rice, wheat, sorghum, millet, corn, and even cotton waste have been reported [29]. Typical spawn loading rates in this method are 10 to 20% of the weight of the substrate [40]. The grain provides a nutrient-rich substrate for the homogeneous and rapid growth of the mycelium [29]. A study on the spawn loading ratio of a millet-based spawn found higher densities associated with higher spawn to substrate ratios, due to the spawn's inherent higher density compared to the substrate [2]. A study comparing wheat and millet as substrates for spawn production demonstrated that the most ideal composition was a 1:1 mix of millet and wheat grain. This improved growth brought by the mixture-spawn compared to each grain type independently may be the product of complementing chemical compositions of the grains, suggesting that strategic grain composition selection could facilitate improved growth rates [29]. The main drawback of grain-based spawn is that it competes with the food and feed supply, and alternatives such as reclaimed cellulose as spawn feed have been reported to combat this challenge [41]. The use of the same biomass type as the substrate may also present benefits,

including the acclimation of the fungal metabolic pathways to the chemical composition of the final matrix, reduction of costs associated with grain use, and the homogeneity of the final matrix.

Liquid spawn is produced through submerged fermentation, also referred to as liquid state fermentation (LSF). Advantages of liquid spawn include rapid colonization and scalability, while its disadvantages include heightened contamination risk and shorter shelf life [16]. Examples of this spawn strategy include the 7-day LSF of *Trametes pubescens* mycelium in potato dextrose agar, under 28°C and shaking at 150 rpm [42]. Spawn loading in this method has been reported at both 20 mL of spawn slurry for 100 g of substrate, and 10 mL of slurry in 500 g of substrate, demonstrating protocol specificity [42, 43]. Relative to SSF spawn, LSF spawn has been reported to yield a material with lower density [2]. An evaluation of the age and concentration of the liquid spawn concluded that 3-day old spawn with 25% concentration of the original inoculum was ideal [41].

Other alternative spawn types have also been reported with distinct benefits. Artificial spawns such as alginate bead encapsulated mycelium spawn simultaneously address both shelf-life related challenges and contamination risk [44]. Practical considerations include that the beads can be maintained frozen for six months without loss of viability in the assayed species, with industrial applicability. When compared in performance to SSF spawn, growth in the substrate remains statistically within the same range, demonstrating no negative impacts on growth associated with the beading technology [44]. Sticks inoculum has also been described to bring easy handling, controlled positioning, and contamination reduction to the process [16]. Furthermore, mycelial biomass that is in anhydrobiosis or has been lyophilized enters a dormant state that can be reactivated upon hydration, acting as a means of transferring mycelium seed to the substrate [45].

Environmental Process Control

The design of the MBC bioprocess includes specific environmental parameters that are dependent on the bioprocess setup. Various setups for the cultivation of the MBC have been reported at different scales. At the laboratory scale, Qiu et al. reported the production of MBCs in an incubator equipped with humidifiers and relative humidity sensors, in which gas exchange was limited to the incubator doors opening [46]. A simpler set up was reported by H. Ahmadi et al who described growing the MBC in covered buckets on screens over a pool of water, with tubes inserted

above the water level for maximized gas exchange [47]. In this following section, the effect of cultivation environmental conditions on MBCs has been reviewed to aid in designing an experimental setup.

Relative Humidity

Ideal humidity levels for white-rot fungi range between 70% and 80%, while brown rot fungi prefer exceeding 95% RH, and soft rot fungi grow optimally in RH ranging between 60% and 75% [16, 48]. A study evaluating the effect of RH ranges of 50-60%, 60-70%, 70-80%, 80-90%, and 90-100% on mycelial growth of three white rot species of *G. lucidum*, *P. ostreatus*, and *Agaricus bisporus* found 80-90% to be the optimal growth condition, with higher values underperforming due to contamination and poor aeration [49].

Temperature

The ideal temperature ranges between 20°C to 30°C and is specific to the species, with adjustments required for maximized growth performance [16]. Temperature impacts enzymatic activity during lignocellulosic degradation, displaying optimal catalytic activity around 25-30°C [20]. Additionally, temperature impacts metabolic activity: Temperatures below 20°C inhibit colonization through declining metabolic activity by reducing cell fluidity, which reduces enzyme kinetics. Temperatures above 28°C on the other hand stimulate sporulation instead of vegetative growth, and in extreme cases, heat stress and protein denaturation, stopping growth [20].

Mycelial colonization releases heat in the substrate, and this heat increases the temperature of finer substrates more rapidly due to limited heat dissipation. This phenomenon is more impactful as the bioprocess scales, where in large molds temperature gradients cause structural defects resulting from uneven growth. Modern bioreactors address this issue by implementing controlled aeration channels or mixing arms, facilitating uniform gas and heat exchange in the substrate [20].

A study on the concurrent effects of temperature and RH found that MBCs grown in high temperature (> 27°C) and RH in the 51-70% range presented the highest density, while MBCs cultivated in lower temperatures (< 24°C) and RH of 71-100% held the lowest density [48].

Aeration

Temperature, RH, and even pH are influenced by aeration, as these parameters are tightly related to fungal metabolism. Respiration in the mycelium is exothermic and without sufficient ventilation, substrate temperatures rise as high as 35°C, limiting growth. On the other hand, excessive airflow limits humidity and dries the surface layer, causing mycelial death [20]. Thus, a setup that co-regulates these parameters is critical.

The regulation of pH is also indirectly affected by aeration. The rate of organic acid production and nitrogen metabolism is influenced by oxygen. Proper aeration prevents the buildup of intermediates, which stabilizes the pH around 5.5-6.5, optimal for enzymatic performance [20].

pH

The optimal substrate pH for the growth of most fungi is in the 5 to 8 range, and matching the substrate pH to the target species is essential [9]. Kashyap et al. investigated the effect of pH levels of 5, 6, 7, 8, and 9 on three species (*P. ostreatus*, *G. lucidum*, *Agaricus bisporus*) and found maximum mycelial colonization at pH 7, with growth declining progressively at pH 8 and then 9, while pH 5 produces minimum growth [49]. The influence of pH is primarily through enzymatic activity and fungal metabolism, as ion solubility and nutrient uptake in fungi are impacted by substrate pH. A near-neutral pH facilitates a synergistic relationship between cellulose and lignin degradation, which is necessary for well-bounded porous MBCs [20]. As the mycelium expands, fungal activity reduces pH through the secretion of organic acids and degradation of carbohydrates and proteins, placing an importance on the role of pH buffers during production, such as calcium carbonate, phosphate buffers, and organic buffers [20, 50]. It is noteworthy that an interaction between temperature and pH has been reported that influences enzyme structure and metabolism. The pH shifts that alter the enzyme protonation state affect temperature tolerance, and high temperatures increase acid accumulation [20]. The role of pH with regards to the mechanical properties of the material has been documented as a determinant in mycelial growth rate and density [2]. Lastly, since the environmental pH is responsible for regulating a cascade of pathways that regulate cell-wall modification, it is theoretically possible to leverage pH adjustments to selectively activate or inhibit specific cell-wall modifying enzymes [30].

Light Exposure

Across the literature reviewed herein, incubation has been consistently conducted under dark conditions. Lack of access to light, along with CO₂ levels and temperature, have been identified as the three factors that facilitate the continued growth of the fungus in its vegetative anamorphic state, preventing fruiting body formation [9]. Light serves as a source of information for fungi, impacting their growth and behavior, such as metabolism and reproduction [51]. A study on the concurrent effects of CO₂ and light on *Schizophyllum commune* mycelium grown in liquid static culture reported that lower densities were obtained under high CO₂ in dark and at low CO₂ in light, relative to mycelium grown under low CO₂ in the dark or high CO₂ in light. Additionally, a 1.7-fold higher Young's modulus was achieved in light compared to dark, when grown at high CO₂ [13]. Furthermore, the mycelium possesses electrogenic responses (in the form of electric pulses) to UV light that can be utilized in the activation of electronic systems within applications such as hybrid biorobotics [45].

Molding procedure

Mold material

Mold material must be compatible with mycelium growth and withstand sterilization or disinfection practices; therefore, the most used materials are synthetic plastics such as acrylics, polypropylene, and silicone [16]. Yaacob et al. used thermoformed plastic molds that were sterilized in a laminar flow hood prior to being filled with the matrix [52]. Alaux et al. emphasized the importance of considering shrinkage percentage when cutting PVC/PE foil to specific dimensions [24]. The use of acrylic clear sheets has been reported by Aiduang et al. for samples used in tensile, flexural, and impact tests [53]. Appels et al. Hand-pressed the pre-grown substrate in PET-G molds [54]. Aside from synthetic plastics, the use of polylactic acid (PLA) bioplastic has been reported in 3-D printed modular mold units with detachable faces, optimized for demolding and deformation prevention [55].

Aside from plastic-based molds, the use of metals, wood and wood-based materials, and biodegradable materials emerges in the literature. The use of steel baking sheets has been reported in the production of mycelium composite panels [56]. Coated plywood is an example of a wood product that has been used in the construction of molds for MBC-brick cultivation [22]. Sustainable and degradable materials such as bamboo or plant fibers have also been described, where the mycelium completely colonizes the mold [57]. Textiles reinforced with natural glue have

been used to produce MBC sandwich beach sandals, removing the need for conventional molds [17]. The MBC itself has also been studied as a sustainable and successful mold material to improve bioprocess sustainability and clean production [58].

Molding technique

Conventional mold filling, which involves packing the substrate and spawn mixture in a mold, can be performed in a single stage or two stages. Nussbaumer et al. cultivated the material in single-stage mode, however the material was flipped once during the process for uniform colonization on both sides [59]. In two-stage incubation, the material is fully demolded and cultivated under controlled conditions in demolded state for surface colonization. In the bioprocess of the study of Appels et al., after 14 days of cultivation in molds, the material was demolded and cultivated for 10 more days under the same conditions [54].

Among other novel approaches to the fabrication of MBCs is 3D printing, which is a mold-free alternative. This approach overcomes the limitations of size and geometry in MBCs, and enables improved oxygen exposure [60, 61]. Limitations to 3D printing include substrate clogging during extrusion, poor print quality, growth halting, and contamination risk [48]. In an alternative bioprocess, psyllium husk gel was formulated to be shootable for vertical deposition without molds [62].

Other Metabolic Considerations

Chitin Expression - Magnesium Supplementation

Chitin, a component of the mycelial cell wall, is the second most abundant high strength natural polymer. The several crystalline forms of this homopolymer are termed α , β , γ , with the first being the most abundant. Alpha-chitin's structure includes microfibrils arranged in an antiparallel manner resulting in high tensile strength [63]. Chitin can be found in several eukaryotic groups, including fungi, in which its biosynthesis is conserved in cellular machinery that facilitate the secretion of linear chitin chains into the extracellular matrix, where the organized assembly of microfibrils takes place [63].

The conserved chitin biosynthesis pathway in fungi uses β -D-glucopyranose, or its stored form as a glycogen or α,α -trehalose as the sugar source. After glycolysis the pathway enters the rate-limiting step of amino sugar biosynthesis, involving amination, acetyl group transfer, and isomerization steps catalyzed by glutamine:fructose-6-p aminotransferase, producing a N-acetyl-

D-glucosamine 6-phosphate intermediate. Next, UDP-N-acetyl- α -D-glucosamine, which is an activated-sugar is produced, followed by the final step of its polymerization reaction to chitin catalyzed by chitin synthase (ChS) [63]. Chitin synthases are present as membrane protein complexes and their genes in fungi have been found in multiple copies. The enzyme has been categorized into families based on the amino acid sequence motifs, and it is the only enzyme that is specifically attributed to chitin biosynthesis [64].

The level of chitin expression is anticipated to play a role in the strength of the hyphal network and the overall composite. ChS is highly expressed at the hyphal tip and contributes to hyphal elongation. Different ChSs have been found in fungi, all of which require a cation as a co-factor. The most common co-factor reported among filamentous fungi is Mg^{2+} , which suggests that supplementation with this cation could facilitate the tunability of MBC properties through affecting chitin expression [64]. Previous investigations of supplementation with magnesium salts were targeted at the uptake of the cation by fungi for nutritional purposes [65]. Sawdust medium was supplemented with 0.5%, 1%, and 5% of different magnesium salts. Among the salts, magnesium sulfate presented mild growth inhibition on *Hypsizygus marmoreus*. This supplementation was most ideal at 0.5% and was not analyzed in relation to chitin expression [65].

Incubation Duration

Incubation time varies significantly across different studies. Growth time is a function of substrate volume and substrate-species compatibility. It has been reported to vary between 5 to 42 days depending on the bioprocess, and can even last as long as 90 days in some cases [9, 42]. Protocols split growth into three common phases of bag colonization, molded growth, and demolded growth. Comparative investigations revealed that while *P. ostreatus* required 14-21 days for complete colonization of a 2 kg bag, *G. lucidum* required a longer time of 21-28 days [40]. Another *P. ostreatus*-based method that included all three phases dedicated 30 days to growth in the bag, followed by 7 days molded growth and 3-5 days demolded growth [14].

The importance of growth time optimization is founded on its effect on the properties of the MBC. Longer incubation periods have been reported to significantly enhance compressive strength, up to 60% under optimal conditions, with minimal impact on dry density or thermal conductivity [66]. On the other hand, shortened incubation reduced tensile strength in *Trametes versicolor*, while this reduction was not observed in *Ganoderma sessile*, indicating species dependency [59]. In the study of Teixeira et al., *Pleurotus eryngii*-based material presented plastic

behavior when grown for 15-days, but demonstrated elastic behavior in 30 and 45-day incubation periods [67]. Therefore, the optimization of incubation period for the species used is critical to avoid over-incubation while meeting the desired properties.

Hydrophobins Expression

Hydrophobins are a group of cell wall proteins in filamentous fungi with functional roles in aerial hyphal formation and attachment to hydrophobic surfaces. These small proteins of about 100 amino acids contain eight cysteine residues in a conserved pattern that result in the hydrophobic structure of the protein [35]. *Schizophyllum commune* MBCs with the deletion of the hydrophobin gene demonstrated a higher water retention property, confirming the role of hydrophobins in composite hydrophobicity, which is significant for genetic engineering strategies [13].

Co-culture Cultivation

Microbial co-culturing has been pursued for the improvement of substrate properties for fungal colonization. The use of non-competitive organisms such as *Bacillus subtilis* or *Trichoderma reesei* has been reported advantageous in the modification of lignocellulosic substrates [20]. Further, the co-cultivation of compatible white-rot mycelia can theoretically present a pathway for achieving unique material properties. For example, co-cultures of *Ganoderma applanatum* and *T. versicolor* have been reported to produce amalgamated hyphal systems with a range of induced compounds [68].

Post-processing Techniques

Upon the completion of cultivation, the most widely used method is oven drying, with temperatures ranging between 40 and 125°C for 2 to 72 hours [16]. The appropriate temperature and drying time depend on the size of the piece of material. This method can contribute to the MBC foam structure, as water evaporation during drying creates air pockets in the material [32]. Improper drying can cause dimensional instability, and over-drying weakens bonding and mycelial adhesion, reducing mechanical strength and stability. A comparison of three drying times of 40, 60, and 80°C revealed that drying at 60°C exhibited the highest flexural strength, flexural modulus, and maximum impact load [43].

Cold-pressing MBCs at room temperature is another post-processing strategy that increases material density by 2-fold over the non-pressed controls [54]. In comparison to non-

pressed material, cold-pressed MBCs appear softer with more flexibility in bending, and exhibit a foamy consistency, suggesting that cold pressing improves MBC pliability [52]. Cold-pressed material relative to non-pressed or heat-pressed MBCs, exhibited the most compact hyphal network with superior characteristics such as highest tensile strength, elongation at break, and modulus, in addition to the lowest water absorption [52].

Unlike un-pressed or cold-pressed materials, heat-pressed MBCs often turn brown in color and present a density of up to 3-fold higher than unpressed materials [54]. Hot-pressing may excessively densify the material and weaken the hyphal structure [52]. A flatter and more compact surface were observed in heat pressed material compared to non-pressed, where the water droplets on the surface of the material remained more spherical and absorbed minimally, indicating improved surface hydrophobicity [31]. Hot-pressing has been further described to cause reduced thermal conductivity and increased compressive strength [10].

Alternative post-processing techniques include air drying gradually in ambient temperature, which offers simplicity and energy-efficiency, desirable for small-scale production [16]. The application of water-repellant coatings has also been reported, favoring bio-based coatings such as plant waxes and oils for their sustainability and renewability. Coatings such as beeswax can cut water absorption up to 26% and prevent mold regrowth, although beeswax's temperature-sensitivity limits the use of this coating to applications that ensure temperature-stability [11].

Applications of MBCs

MBCs hold diverse industrial applications for their capability in meeting the mechanical characteristics of both natural materials like wood panels or cork, and synthetic materials like Expanded Polystyrene (EPS). With applications in the civil engineering industry, packaging, automotive sector, aerospace industry, fashion, furniture and interior design, environmental remediation, biomedical applications, electronics, agriculture, and the energy sector, the potential of the material is high.

In the construction and buildings sector, MBCs have been used as thermal and acoustic insulation, bricks and masonry, panels and facades, sandwich composites with MBC core, and even sprayable and 3D printed construction materials. The strongest upward trend in this sector is associated with mycelium bricks, with building blocks presenting a trajectory towards becoming

mainstream in construction systems that aim to comply with the upcoming strict energy efficiency regulation [7]. MBC bricks as thermal insulation reduce temperature fluctuations, as their higher thermal inertia stores more heat and releases it slowly, which reduces peak cooling and heating loads [10]. Application of MBCs as thermal and acoustic insulation is well-established in the literature, reporting exceptional properties based on its low thermal conductivity, porous structure, and good mechanical strength [10]. Sound absorption coefficients were reported to range between 0.33-0.49 for MBCs produced using *P. ostreatus* [69]. Low thermal conductivities (below 0.085 W/m.K) have been reported for MBCs in the literature, strengthening the case for MBC thermal insulation [10]. MBCs as traditional jaali, a perforated passive cooling system, combine thermal insulation with architectural expression [8]. Sprayable MBCs have a curing time of 20-days and can achieve compressive strength values up to 1.51 MPa [62]. Furthermore, the porous structure of MBCs is advantageous for air purification and improvement of indoor air quality [10]. Example species used as insulation include *Lentinus tigrinus* and *Daedalopsis tricolor*, adjusted to the substrate selected [25, 70]. Long-term performance and durability are challenges for the application of MBCs in civil engineering applications. The rapid moisture absorption of these materials can cause mechanical weakening and structural instability [7].

Packaging applications are commercially mature and deployed, pioneered by Ecovative Design. In 2023, the MBC packaging market was valued at USD 74 million and projected to grow to USD 187 million [43]. MBCs have been studied as protective cushioning, with energy absorption values of 35-42 kJ.m⁻³ and cushioning coefficients ranging between 4.7-8, comparable to EPS [42]. MBC furniture pieces have also been proposed, such as the Mycelium Chair and MushLume lighting [55, 61].

The application of MBCs in the automotive and aerospace industries has also been described. A review of MBC patents by Cerimi et al. reported a total of 8 patents in the automotive sector granted to Ford LLC for the manufacturing of the material, up to the year 2018 [57]. The potential of MBCs in space habitat design was pioneered by NASA in 2020, through the exploration of its application in living quarters for future lunar and Martian developments. Benefits of the use of MBCs as the inner layer of the habitat structures include self-replication and on-site growth, self-healing, sensing properties, waste reduction, radiation protection, and psychological benefits [61].

Less prominent applications are also worth mentioning. Ahmadi et al. evaluated the use of 25-days old living and dried MBCs from *Pleurotus djamor* and cellulose microfibers on the

remediation of leaking potassium hydroxide alkaline battery waste, demonstrating the application of MBCs in bioremediation [47]. Floating planting beds made of mycelium biomaterial are another example of the bioremediation potential of the material, where it cleanses waterways of pollutants and remediates wetlands [61]. Biomedical applications have also been described. Cotton-based MBCs produced through submerged fermentation were found to possess antibacterial activity when tested against both gram-negative and gram-positive pathogens, due to MBC's chitin and glucan content [71]. Lastly, electronics applications include the use of MBCs in circuit boards and MBC-based batteries [61].

Limitations of MBCs

Limitations of MBCs continue to hinder industrial adoption and public acceptance. With regards to their mechanical properties, although compressive strength varies depending on the protocol, it typically ranges between 0.05 to 0.18 MPa, which is low compared to conventional materials [7]. Additionally, the low elastic moduli and foam-like properties of these materials limit their application in civil engineering to non-structural or semi-structural uses, such as insulation, paneling, and furniture [7, 32]. The inherent hygroscopicity of MBCs, reported to exceed 200% by weight in certain findings, can be a limitation depending on the specific end-use [7]. The natural biological variability of the mycelium in such grown materials results in the inconsistency of derived properties, impeding protocol standardization, and inspection methodologies [12]. Although small-scale production creates market-entrants, large-scale production remains a challenge as MBC production requires days to weeks for production, unlike synthetic materials that are manufactured in hours. Contamination risks and environmental fluctuations further undermine scalability [12]. The limited lifespan of MBCs, reported as less than 50 years, presents a durability challenge for long-term applications such as those in the civil engineering industry [32]. Without protective coatings, the material is susceptible to weathering, mold or bacterial colonization, and even insect or pest infestation [12]. Shaped designs also face mycelial colonization limitations caused by fiber size and oxygen access, which further restricts potential end-uses [56]. Health and safety risks also persist, as some fungi may be allergenic producing mycotoxins, attract insects, or become invasive [9]. Lastly, consumer and industry adoption requires a broad social shift to bring forward acceptance of the use of fungi in materials [72].

Conclusions

The fabrication and applications of MBCs have been reviewed in this chapter to guide experimental design. The technoeconomic and environmental case made for MBCs across the literature has been summarized. Each fabrication condition has been evaluated for its effect on the yielded material. This chapter serves as a foundation for pursuing the expansion of the body of existing work on MBCs, which is necessary for the advancement of the material.

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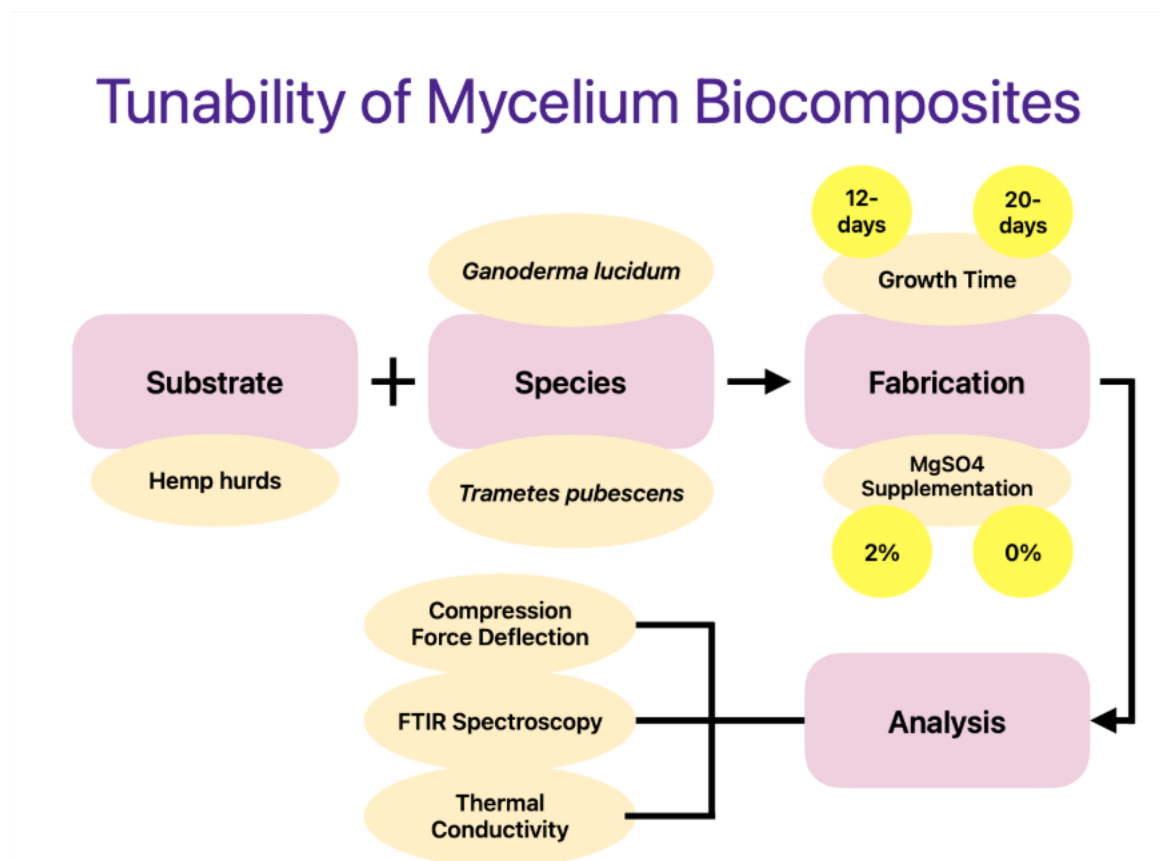
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Chapter 2: The Impact of Magnesium Sulfate Supplementation and Growth Duration on the Properties of Mycelium-based Composites and Chitin Expression

Abstract

Mycelium-based composites (MBCs) are eco-friendly renewable alternatives to fossil-fuel based foams and composites, bringing forward resource efficiency, biodegradability, carbon sequestration, and green bioengineering. Produced from agricultural waste and fungal mycelium, the physical and mechanical properties of MBCs vary depending on the growth conditions of material production, after the choice of the substrate and fungal species. The tunability of the key properties of hemp-hurds based MBCs was investigated by varying growth time and supplementation with MgSO_4 using *Ganoderma lucidum* and *Trametes pubescens*. The properties of the MBCs were investigated through unconfined compression force deflection, Fourier transform infrared (FTIR) spectroscopy, transient thermal conductivity measurement, and various measurements of physical characteristics. Compressive strength at 10% strain ranged between 43.11-73.23 kPa, with the highest strength belonging to the *G. lucidum* magnesium supplemented short-cultivation treatment. The thermal conductivity of the samples differed minimally within the 0.0495 to 0.055 $\text{W}\cdot\text{m}^{-1}\cdot\text{K}^{-1}$ range. FTIR analysis suggested that MgSO_4 supplementation may increase mycelial chitin content in a species-dependent manner. Overall, *T. pubescens* produced composites with lower density and Young's modulus but higher water absorption rate (WAR), characteristics that may be advantageous for certain packaging applications. *G. lucidum* delivers a biocomposite with higher compressive strength and low WAR, ideal for applications in the construction industry.

Graphical Abstract



Introduction

The advancement of circular economy in support of net zero strategies requires the exploration of innovative sustainable materials as alternatives to those with high greenhouse gas (GHG) footprint [1]. Mycelium-based composites (MBCs) are bio-degradable foam-like composite materials produced from renewable agri-forestry waste and the fungal mycelium, with properties comparable to fossil-fuel based foams and composites. Although subject to limitations, the mechanical properties of MBCs enable competition with wood composites that utilize non-renewable synthetic binders as well as fossil fuel-based foams such as expanded polystyrene (EPS). The reuse of carbon captured as renewable biomass as raw material for MBC production delays emissions associated with biomass decomposition, while simultaneously replacing emissions from high-footprint foams and composites. Studies have reported that MBCs can provide over 90% cost savings relative to wood composites and polymer materials [2]. Therefore,

the expansion of the production and commercialization of MBCs could improve economic and environmental circularity and sustainability.

The mechanical properties of MBCs display great variability based on fungal species and fabrication conditions, and gaps exist on how to deliberately tune their properties [3]. Due to the complexity of living organisms, the adjustment of the MBC bioprocess can produce unique material properties, enabling the tunability of MBCs. Although this tunability in stiffness, strength, elasticity, and water resistance has been conceptualized, expanded controlled studies are needed to link specific cultivation parameters with quantifiable changes in mechanical properties [4]. Specifically, the effects of primary biological factors such as cell-wall chemistry and growth stage remain underexplored and have been addressed herein.

In MBCs, the mycelial tissue acts as a natural binding agent, replacing the demand for hazardous synthetic binders in composites. Mycelium is comprised of hollow structures named hyphae that form the root-like vegetative tissue in higher fungi. The hyphal network can be composed of generative, skeletal, or ligative hyphal types [2]. Fungi can be monomitic, dimitic, or trimitic depending on whether their mycelial network is composed of one, two, or all three types of hyphal structures, respectively. A distinct correlation between hyphal type and mechanical properties of sporocarps has been found where compression modulus and compressive strength increase under the presence of more hyphal types in the mycelial system [5]. Furthermore, in trimitic organisms, a higher percentage of hyphal volume is composed of fungal-wall, indicating the presence of a thicker cell-wall compared to mono- or dimitic organisms [6]. The thicker cell wall increases hyphal stiffness and may contribute to improved mechanical performance in mycelium-derived materials [6]. Founded on these considerations, this study has focused on the performance of two trimitic basidiomycetes, *Ganoderma lucidum* and *Trametes pubescens*, as MBC binders.

Production of an eco-friendly MBC with desirable properties requires the selection of a substrate that is both sustainable and capable of delivering the desired mechanical characteristics. In this study, hemp hurds were selected as the substrate on both grounds. Comparison of the thermal conductivity of MBCs produced from chopped hemp, flax, and straw fibers placed hemp to be the substrate that provided greater thermal insulation, desirable for civil engineering applications [7]. On the other hand, in Canada, the growing hemp agriculture industry creates a renewable stream of hemp hurds, presenting the opportunity for its sustainable

valorization [8]. With over 150 million tons of hemp waste produced annually, the global impact of hemp waste valorization is significant [9]. The reuse of the CO₂ captured as hemp by-products in a material that can replace high-footprint foams and composites falls among the carbon management processes described under Canada's Carbon Management Strategy [10].

The two fabrication conditions studied for material tunability were supplementation with MgSO₄ (Epsom salt) and substrate growth time. Firstly, MgSO₄ provides a source of magnesium and sulfur for the fungus, and the study of its effect on *Cordyceps militaris* positioned it as a preferred magnesium source [11]. Magnesium plays a broad role in oxidation processes and is critical to the function of certain enzymes and in respiration [11]. The Mg²⁺ cation is a cofactor to the enzyme Chitin Synthase (ChS), which is responsible for the synthesis of the strong natural polymer chitin [12]. Chitin, a component of the mycelial cell wall, is the second most abundant high strength organic polymer after cellulose [13]. Chitin-producing fungi are equipped with a conserved ChS-dependent cellular mechanism through which sugars are converted into linear chitin chains and secreted to the extracellular cell-walls [13]. It was hypothesized that supplementation with MgSO₄ may influence ChS-mediated chitin biosynthesis through increased cofactor availability, increasing chitin content of the mycelial cell-wall. Increased chitin levels in the mycelium were expected to concurrently promote material strength for the mechanical properties of chitin, and reduce water absorption for the hydrophobic behavior of this polymer. Interestingly, magnesium sulfate heptahydrate has been reported to be an effective flame-retardant when supplemented in hemp shiv composites for its cooling and gas dilution effects, strengthening the case for its use in MBCs [14]. To the best of our knowledge, this is the first report of the effects of MgSO₄·7H₂O (Epsom salt) as a supplement on MBC properties.

The second fabrication condition studied was the duration of substrate growth. Previous investigations reported conflicting results on physical and mechanical properties in relation to growth time [15, 16]. Increased growth time is anticipated to result in higher mycelial growth, increasing porosity, and reducing density. The fungal mycelium is expected to express different amounts of various compounds (metabolites, enzymes) and endure varying degrees of substrate degradation depending on its age, altering the chemical composition of the MBC. Here, the cultivation time was divided into four phases of which the third phase of substrate growth was varied in duration between 12 days and 20 days, while the durations of the other phases (inoculant, spawn, and composite) were kept constant.

Methods

Cell Line Maintenance

G. lucidum (WIN(M) 1804) and *T. pubescens* (WIN(M) 1801) were obtained from the Department of Microbiology at the University of Manitoba (WIN(M) collection). Fungal strains were maintained on Potato Dextrose Agar (PDA) slants and stored in the refrigerator at 4° C. Mycelial colonies were transferred to plates of 2% w/w hemp hurds and PDA media to activate mycelial growth when needed for inoculant production.

Experimental Design

The experimental structure involved two process factors, MgSO₄ supplementation and substrate growth duration, applied to two white-rot fungal species of *Ganoderma lucidum* and *Trametes pubescens*.

Table 1. Experimental design and treatment conditions.

Treatment	Substrate Growth Cultivation # of days	Spawn Cultivation – solid state fermentation	Magnesium sulfate supplementation (2% w/w)
T1 – GL, SC, 0%Mg	12	<i>Ganoderma lucidum</i>	No
T2 – GL, LC, 0%Mg	20	<i>Ganoderma lucidum</i>	No
T3 – GL, SC, 2%Mg	12	<i>Ganoderma lucidum</i>	Yes
T4 – GL, LC, 2%Mg	20	<i>Ganoderma lucidum</i>	Yes
T9 – TP, SC, 0%Mg	12	<i>Trametes pubescens</i>	No
T10 – TP, LC, 0%Mg	20	<i>Trametes pubescens</i>	No
T11 – TP, SC, 2%Mg	12	<i>Trametes pubescens</i>	Yes
T12 – TP, LC, 2%Mg	20	<i>Trametes pubescens</i>	Yes

Note: Acronyms are as follows: GL = *Ganoderma lucidum*, TP = *Trametes pubescens*, LC = Long cultivation, SC = Short cultivation.

Mycelium Comparison

Potato dextrose agar (PDA) plates supplemented with 2% w/w hemp hurds were inoculated with pure mycelial tissue of *G. lucidum* and *T. pubescens*. After ~6 days of growth, the productive zone of the mycelial colony was imaged using a Zoom Microscope (AmScope, USA) at 8x magnification. Images were edited to maximum contrast at 100% and 20% increased brightness for optimal visualization.

Radial Growth Rate

The radial growth of the mycelium of both species was measured on inoculated petri dishes containing PDA and 2% w/w hemp hurds under both no MgSO_4 and 0.4% w/w MgSO_4 supplementation over 8 days (192 hours). Colony diameter was measured daily along two perpendicular axes, and the rate was measured for two replicates of each condition, reported in mm/h.

Inoculant Production

Cell lines were transferred from the activated media plates containing PDA and 2% hemp hurds to fresh plates of same with or without supplementation with 0.4% MgSO_4 depending on the MgSO_4 requirement of the respective treatment. The 0.4% MgSO_4 supplementation concentration in this stage was selected to facilitate the acclimatization of the fungi to the supplement, to curb inhibitory responses. Colonies were maintained on the plates for a duration of 8-12 days inside an incubator set at 23°C, until complete plate coverage was achieved, upon which this media and mycelium biomass - referred to as the inoculant (25-35g total) - were transferred for spawn production.

Spawn Production

60 grams of hemp hurds at 85% moisture content, with or without 2% w/w MgSO_4 depending on the treatment, were autoclaved at 121°C for 20 minutes and cooled to room temperature. For solid state spawn production, the inoculant was transferred to these bags and mixed within the substrate to complete homogenization. The spawn was grown for 9 days in an incubator set at 23°C and agitated every 48-72 hours to the point of complete disruption of the mycelial network.

Substrate Cultivation

150 grams of hemp hurds substrate at a moisture content of 85% with or without 2% w/w MgSO_4 supplementation depending on the treatment were autoclaved in large autoclavable bags, cooled to room temperature, and inoculated with 100 grams of 9-day-old spawn. Depending on the treatment, the substrate cultivation phase lasted 12 or 20 days, inside an incubator at 23°C and agitated every 48-72 hours to complete homogenization.

Composite Formation and Cultivation

Composite formworks and associated equipment were exposed to UV sterilization for 1-hour by exposure to a UV-C lamp inside a biosafety cabinet (Air Clean Systems, USA), inside which composite-shaping also took place to minimize contamination. Twenty-seven grams of homogenized mycelium-substrate matrix was packed densely at maximum compaction into food-grade polypropylene formworks/molds with dimensions of 75 x 75 x 25 mm. All sides of the sample were covered with polypropylene material, and the surfaces contained a few small holes to facilitate airflow to the mycelium. The shaped hemp-mycelium matrix was incubated in a humidified incubator (Thermo Fisher Scientific, USA) at 85% Relative Humidity and 23°C for 9 days. After the 9-day composite incubation, the samples were separated from the formworks and oven dried at approximately 90°C for 24 hours.

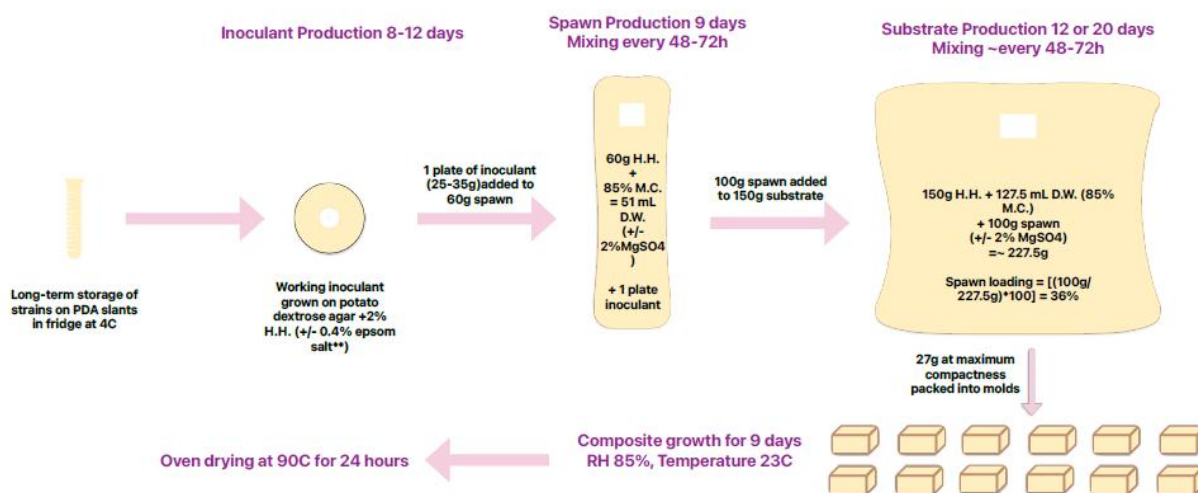


Figure 1. Schematic of the laboratory-scale fabrication process. Acronyms are as follows: M.C. = moisture content, D.W. = deionized water, H.H. = hemp hurds, PDA = potato dextrose agar. RH = relative humidity.

Physio-Mechanical Characterization

Compression Force Deflection (CFD)

A standard triaxial load frame (Wykeham Farrance, UK) was used to test triplicate samples in each treatment for comparing the compressive strength, Young's modulus, and recovery properties of the samples. Dry samples were deformed to 75% of their original height at a deformation rate of 5 mm/min and a sampling rate of 2 Hz to generate stress-strain curves. Compressive strengths at 10% and 35% deformation, and the Young's modulus of each sample were determined. Young's modulus was calculated as the slope of the linear portion of the stress-strain curve (up to 50% deformation). To measure recovery, the height of the samples was measured after compression upon rest intervals of 1, 3, 5, 10, 20, 30, and 60 minutes. The recovery rate was then calculated as a percentage:

$$Recovery = \frac{H_f}{H_0} \times 100 \quad (1)$$

Where H_0 was the height immediately after removing the load, and H_f was the height after the assigned rest period.

Thermal Conductivity

The ASTM D5334 Standard method for determination of Thermal Conductivity of Soil and Rock by Thermal Needle Probe Procedure is commonly adapted for wood-based composites and was followed for the analysis of thermal conductivity of MBCs [17]. A KD2 Pro Thermal Properties Analyzer (Decagon Devices Inc., USA) was used to conduct the transient line heat source method of measurement using the KS-1 single needle probe with dimensions of 1.2 mm diameter and 60 mm length. Two specimen blocks were fixed on top of each other using rubber bands, and the needle was inserted between them with intimate contact to surface. Duplicate readings were measured and averaged from the two blocks by inserting the needle 90° apart for each reading.

Dry Density

Dry density was calculated by dividing the total dry weight of the samples to the measured dry volume of the samples. For all samples in each treatment, the dry weight was measured immediately after oven drying using a standard balance scale. The length and width of each sample

was reported as an average of four measurements taken from each side of the sample (90° apart) using digital calipers.

$$\rho = \frac{m}{v} \quad (2)$$

Where m is the dry weight of the sample, and v is the dry volume.

Moisture Content

The moisture content was evaluated by measuring the weight of the samples before and after the post-production oven drying, and was reported as a percentage based on formula 3:

$$M.C. = \frac{(M_w - M_d)}{M_w} \times 100 \quad (3)$$

Where M_w is the wet weight and M_d is the dry weight of the samples.

Shrinkage Percentage

The Shrinkage Percentage was calculated as the percentage of reduction in volume before and after oven drying.

$$Shrinkage = \frac{(V_w - V_d)}{V_w} \times 100 \quad (4)$$

Where V_w is the wet volume of the samples and V_d is the dry volume.

Water Absorption Rate and Volumetric Swelling Rate

An adaptation of the water absorption and thickness swelling method in the standard test methods for evaluating the properties of wood-based fibers and particle panel materials in ASTM D1037 was used [18]. Triplicate MBC samples from each treatment were immersed in deionized water at room temperature. The weight and size dimensions of the samples were measured after 1, 2, 4, 8, 12, 24, 48, and 72 hours. Water absorption rate was calculated as follows:

$$W.A.R. = \frac{(W_f - W_i)}{W_i} \times 100 \quad (5)$$

Where W_i is the initial dry weight of the sample and W_f is the wet weight after the period of immersion.

For volumetric swelling, four measurements (90° apart) were taken from each sample side for thickness and length, and the averages were used to calculate the sample volume. The volumes after each immersion time point were used to calculate volumetric swelling rate:

$$V.S.R. = \frac{(V_f - V_i)}{V_i} \times 100 \quad (6)$$

Where V_i is the initial dry volume before immersion, V_f is the volume after the immersion period.

Fourier Transform Infrared (FTIR) Spectroscopy – Transmission Mode

Samples were re-dried at 90° C for 24 hours prior to FTIR spectroscopy to ensure no absorbed moisture during storage interfered with the spectra. Approximately 1 gram of the sample corner was ground to powder using mortar and pestle to ensure a representative sample of all components was captured. Precisely, 5 mg of sample powder was weighed and homogenized with 145 mg of potassium bromide (KBr) until uniform, after which it was pressed under 10 tons of pressure in a laboratory press (Spex 3624B X-PRESS, USA) for 1 minute to form a pellet. Using a Bruker Tensor 27 FTIR spectrometer, absorbance spectra were acquired at a resolution of 1 cm⁻¹. The spectra for three biological replicates in each treatment were analyzed, and the spectral data were averaged for visual presentation of the treatments.

FTIR Semi-Quantitative Analysis Model

Preprocessing of FTIR data was performed to reduce noise and enhance signals. The preprocessing involved the normalization of the FTIR spectra for comparability, which addresses the effect of varying pallet thickness and density across samples. Each replicate was normalized to the total integrated area of the fingerprint region (1800-800 cm⁻¹) as a normalization factor [19]. The total area of the fingerprint region was calculated via trapezoidal integration in excel.

The bands observed in the FTIR spectra were assigned to functional groups, and the compounds expected to present the identified vibrations were inferred from the functional groups. The regions of the spectra where the contribution of lignin, cellulose, chitin, or mycelium was most notable were identified and the area of these regions calculated via trapezoidal integration was assigned as Lignin Index, Cellulose Index, Chitin Index, and Mycelium Index, respectively.

To determine a mycelium to cellulose ratio for each treatment, Mycelium Index divided by Cellulose Index was calculated. This ratio for each treatment was subtracted from the value of the

ratio for pure hemp hurds, and the product was assigned as the Growth Index. Since this ratio is based on the region assigned to mycelium's contribution and cellulose's contribution to the composite, it reflects the amount of mycelial growth having taken place, where cellulose reduces through degradation and mycelial biomass increases.

$$\text{Growth Index} = \left(\frac{\text{Mycelium Index}_{\text{sample}}}{\text{Cellulose Index}_{\text{sample}}} \right) - \left(\frac{\text{Mycelium Index}_{\text{hemp}}}{\text{Cellulose Index}_{\text{hemp}}} \right) \quad (7)$$

Two ratios of Composite Chitin Index and Mycelium Chitin Index were calculated using the Chitin Index. The ratio of Chitin Index to Cellulose Index of each sample subtracted from that of hemp hurds exhibits a quantitative metric to compare chitin's contribution to the whole biocomposite. Chitin has been previously used as a determinant of estimated mycelial growth [20]. Therefore, the Composite Chitin Index provides a relative comparison of the mycelial density of the different cultivation conditions.

$$\text{Composite Chitin Index} = \left(\frac{\text{Chitin Index}_{\text{sample}}}{\text{Cellulose Index}_{\text{sample}}} \right) - \left(\frac{\text{Chitin Index}_{\text{hemp}}}{\text{Cellulose Index}_{\text{hemp}}} \right) \quad (8)$$

To derive a value that describes the amount of chitin per unit of mycelium, informing on the cellular effects of MgSO_4 supplementation, the Mycelium Index was divided by the Chitin Index for each sample and subtracted from that of pure hemp hurds. The result was assigned to be the Mycelium Chitin Index, a value reflective of the relationship between the mycelium composition of a sample and the chitin composition in relation to that, reflecting chitin per unit of mycelium.

$$\text{Mycelium Chitin Index} = \left(\frac{\text{Mycelium Index}_{\text{sample}}}{\text{Chitin Index}_{\text{sample}}} \right) - \left(\frac{\text{Mycelium Index}_{\text{hemp}}}{\text{Chitin Index}_{\text{hemp}}} \right) \quad (9)$$

Because FTIR spectra of lignocellulosic-fungal composites contain overlapping contributions from multiple biochemical constituents, the derived indices should be interpreted as relative spectral indicators rather than direct quantitative measurements of chitin concentration.

Statistical Analysis

Three-way factorial ANOVA ($\alpha = 0.05$) was conducted for the results of compressive strength, Young's modulus, density, Growth Index, Composite Chitin Index, and Mycelium Chitin Index. The main effects were species, growth duration, and MgSO_4 supplementation, and interactions between the factors were evaluated. The results of compressive strength and Young's modulus were further analyzed via post-hoc Tukey HSD.

Two-way ANOVA ($\alpha = 0.05$) was conducted for the results of Water Absorption Rate data of each species independently, due to the confounding effect of species. The main effects were growth duration and MgSO_4 supplementation, and interactions were evaluated.

Results

Mycelium Structure

Species-dependent MBC characteristics are driven by the hyphal network of the mycelia. To examine the hypha, the productive zone of the mycelial plates of *G. lucidum* and *T. pubescens* were imaged and exhibited in Figure 2. Both organisms possess trimitic hyphal networks, where the mycelium is comprised of a mixture of all three hyphal types of skeletal, generative, and binding hypha. Despite this similarity, the strain of *G. lucidum* used in this study exhibited comparatively straighter and less branched hypha, consistent with the predominance of skeletal hypha. In contrast, the morphology of *T. pubescens*'s mycelium with frequent branching suggests higher contribution of generative and/or ligative hypha.[6].

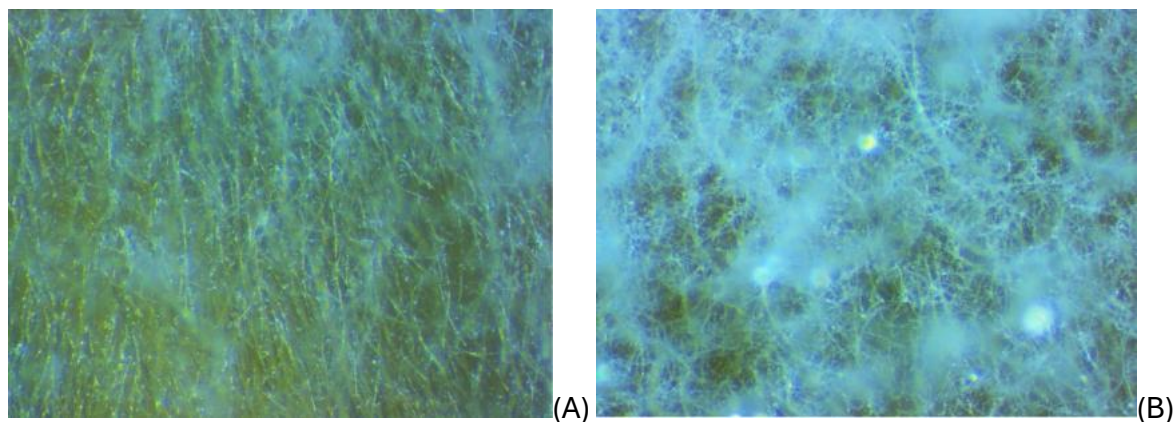


Figure 2. Zoom microscopy images of the productive zone of mycelial colonies on media plates of potato dextrose agar supplemented with 2% hemp hurds. (A) *G. lucidum* (B) *T. pubescens*.

Mycelium Growth Rate

Radial growth measurements revealed that *G. lucidum* generally possesses a higher mycelial growth rate in comparison to *T. pubescens* when grown as a single colony on a petri plate. No significant enhancement or inhibition of growth was observed under media supplementation with 0.4% w/w MgSO_4 .

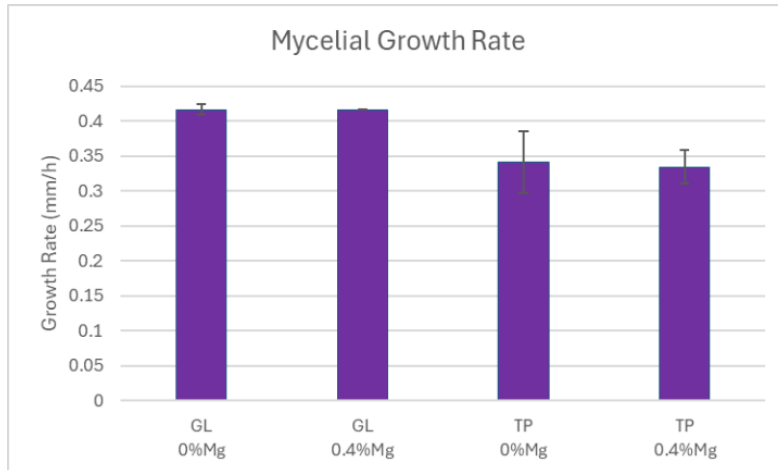


Figure 3. Radial growth of the mycelium of *G. lucidum* (GL) and *T. pubescens* (TP) under no supplementation with Epsom salt (0%Mg) and 0.4% w/w supplementation with Epsom salt (0.4%Mg). Error bars indicate standard deviation.

Mechanical Properties

Compressive Strength

Unconfined CFD tests generated stress-strain curves, from which the compressive strengths of the MBCs were determined at 10% and 35% deformation as the average stress at that range in kPa, presented in Table 2.

Table 2. Compressive strength of the materials. GL = *Ganoderma lucidum*. TP = *Trametes pubescens*. S.C. = Short Cultivation. L.C. = Long Cultivation.

Treatment	Compressive strength at 10% strain (kPa)	Compressive strength at 35% strain (kPa)
T1 – GL, S.C. No MgSO ₄	69.49+/-9.0	423.44+/-26.5
T2 – GL, L.C., No MgSO ₄	47.51+/-6.8	319.72+/-32.9
T3 – GL, S.C., 2% MgSO ₄	73.23+/-13.5	452.31+/-56.4
T4 – GL, L.C., 2% MgSO ₄	57.63+/-5.6	403.48+/-26.1
T9 – TP, S.C., No MgSO ₄	43.11+/-6.4	257.10+/-12.6
T10 – TP, L.C., No MgSO ₄	53.96+/-3.2	256.78+/-9.2
T11 – TP, S.C., 2% MgSO ₄	61.98+/-9.2	297.61+/-5.1
T12 – TP, L.C., 2% MgSO ₄	55.66+/-9.4	264.85+/-8.4

In the three-way factorial ANOVA of compressive strengths, species, substrate growth time, and MgSO_4 supplementation were kept as fixed factors. At 10% strain, species had a significant effect on compressive strength, $p = 0.0280$, with GL-MBCs showing a higher marginal mean strength than TP-MBCs. Growth duration was also significant, $p = 0.0285$, with short cultivation producing a higher mean strength than long cultivation. MgSO_4 supplementation also significantly increased compressive strength at 10% strain, $p = 0.0233$. The Species x Growth Time interaction was the only significant interaction ($p = 0.0073$), indicating that the effect of cultivation duration differed between GL and TP. A similar but stronger pattern was observed for compressive strength at 35% strain, where the effect of species ($p = 2.99\text{E-}9$), growth duration ($p = 0.0007$), MgSO_4 supplementation ($p = 0.0024$), and the interaction of Species x Growth time ($p = 0.0167$) were significant. It is noteworthy that the interactions involving MgSO_4 supplementation were not statistically significant, which is important because it suggests that the positive effect of supplementation remained consistent across both species and growth durations.

Overall, GL-MBCs produced higher compressive strengths than TP-MBCs, confirming the strong influence of fungal species on mechanical performance. MgSO_4 supplementation also presented a significant positive effect on compressive strength, although Tukey HSD comparisons revealed that not every individual supplemented treatment differed significantly from its unsupplemented counterpart. Short cultivation generally produced stronger materials than long cultivation, but the significant Species x Growth Time interaction suggests that this effect was more pronounced in GL-MBCs than in TP-MBCs. Therefore, the compressive strength results suggest that MgSO_4 supplementation generally improves mechanical performance, while the effect of growth duration depends on the fungal species.

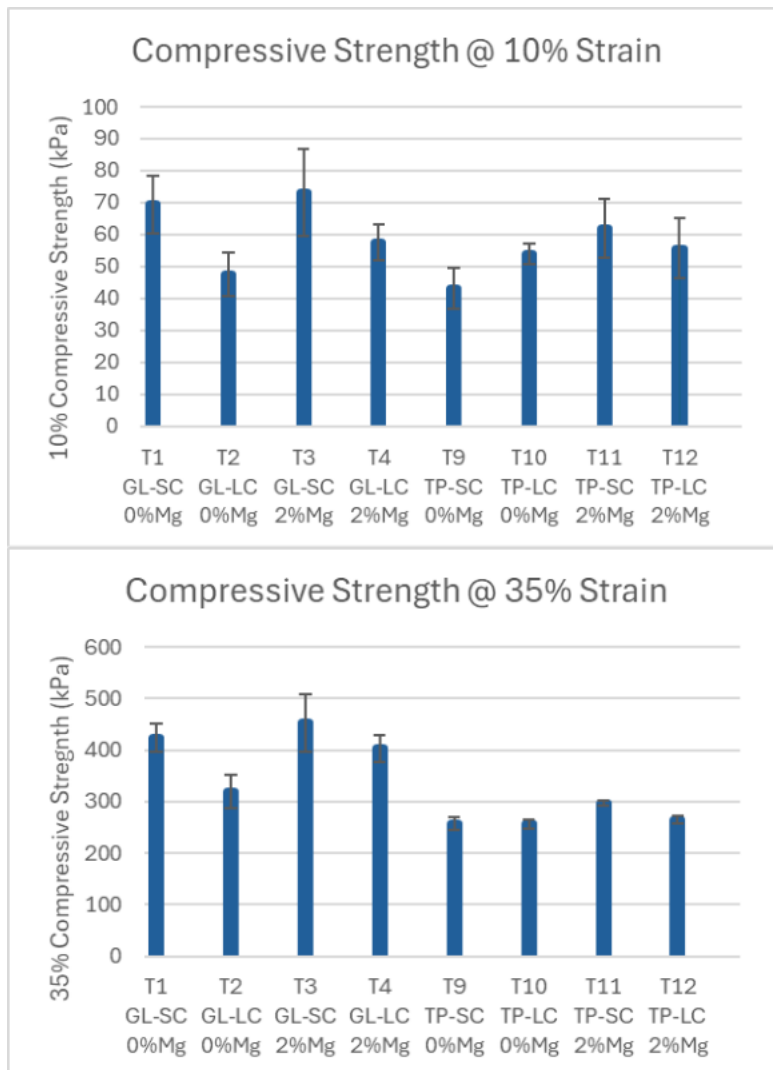


Figure 4. Compressive strength of the samples at 10% and 35% deformation, calculated from stress-strain curves obtained through unconfined CFD tests.

Young's Modulus

The compressive (Young's) modulus, which reflects the stiffness of the material, has been calculated as the slope of the linear portion (up to 50% strain) of the stress-strain curves.

Generally, the MBCs produced by *G. lucidum* are stiffer materials compared to the MBCs produced by *T. pubescens*, potentially reflecting the morphological role of the predominance of skeletal hypha in *G. lucidum*. Within the three-way ANOVA, species had a significant effect on Young's modulus ($p = 4.92E-10$), with GL-MBCs showing a higher marginal mean modulus than TP-MBCs. Growth duration was also significant ($p = 0.0002$), with short cultivation producing higher moduli than long cultivation. MgSO₄ supplementation also significantly increased Young's modulus ($p = 0.0028$). The Species x Growth Time interaction was also significant, following the trend seen in

compressive strength ($p = 0.0343$), which indicates that the effect of growth duration differed between the two fungal species. The other two-way and the three-way interactions were not of statistical significance.

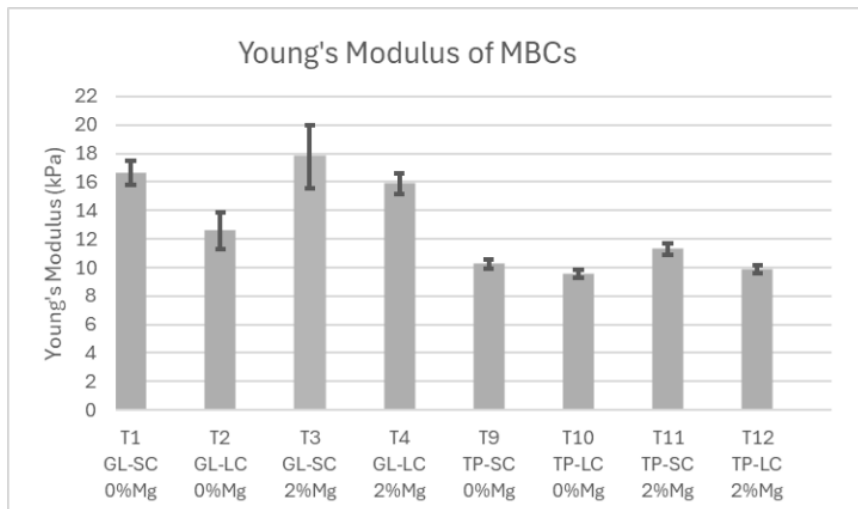


Figure 5. Young's modulus of MBCs of the eight treatments.

Recovery Property

The recovery property, which reflects the MBC's ability to carry secondary load, can be useful for applications such as packaging. The internal structure of MBC possesses resiliency towards deformation, demonstrating elastic recovery in the 36-55% range after 60 minutes. Most of the recovery occurs during the first minute after the load is removed and approaches a plateau at about 20-30 minutes. This suggests that the cell-wall and mycelial network's elastic rebound bring about immediate recovery, and the secondary slower recovery is associated with viscoelastic relaxation. Despite this elasticity, the material also undergoes considerable permanent deformation.

Table 3. Recovery property of samples after 75% deformation under load.

Treatment	Recovery % after 1 min	Recovery % after 3 min	Recovery % after 5 min	Recovery % after 10 min	Recovery % after 20 min	Recovery % after 30 min	Recovery % after 60 min
T1	29.79 +/- 0.9	30.28 +/- 1.3	32.77 +/- 2.7	33.29 +/- 1.3	35.34 +/- 0.9	36.36 +/- 1.4	36.40 +/- 0.9
T2	47.31 +/- 2.9	50.05 +/- 1.7	51.20 +/- 1.4	52.23 +/- 1.7	52.97 +/- 1.1	54.05 +/- 0.4	54.78 +/- 0.8
T3	45.61 +/- 2.0	47.46 +/- 1.7	49.33 +/- 1.1	50.10 +/- 1.5	50.79 +/- 1.3	51.25 +/- 1.4	51.82 +/- 0.6
T4	46.94 +/- 2.6	49.77 +/- 3.7	49.70 +/- 2.3	50.09 +/- 2.5	50.91 +/- 1.4	50.96 +/- 1.9	51.31 +/- 2.7
T9	47.55 +/- 1.7	49.55 +/- 1.8	50.22 +/- 1.7	50.77 +/- 1.1	51.79 +/- 1.3	52.11 +/- 1.0	53.71 +/- 1.0

T10	49.63 +/- 2.4	44.12 +/- 9.2	46.45 +/- 8.4	46.84 +/- 7.7	49.39 +/- 7.5	48.93 +/- 7.7	49.89 +/- 8.4
T11	47.81 +/- 0.4	49.47 +/- 1.5	50.06 +/- 1.4	50.71 +/- 0.9	52.40 +/- 1.2	52.83 +/- 0.7	53.14 +/- 0.8
T12	48.28 +/- 1.3	49.95 +/- 1.1	51.19 +/- 0.3	52.43 +/- 1.3	53.27 +/- 0.4	54.12 +/- 0.7	54.79 +/- 0.4

Thermal Conductivity

The internal structure of the material, which is primarily defined by substrate characteristics, governs heat and mass transfer processes that dictate material thermal conductivity [21]. Here, thermal conductivity varied negligibly when compared across the MBCs of different cultivation conditions, indicating that the effect of species, MgSO_4 supplementation, and growth time are negligible on thermal conductivity.

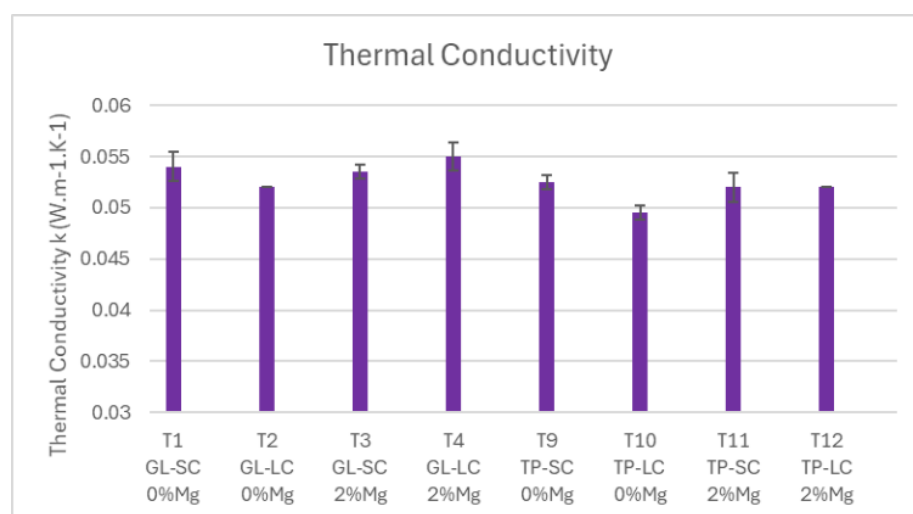


Figure 6. Thermal Conductivity of various cultivation conditions of hemp hurds-based MBC.

Chemical Composition

Fourier Transform Infrared Spectroscopy

The spectra were normalized to the area of the fingerprint region between 1800 and 800 cm^{-1} , and have been presented in Figure 7.

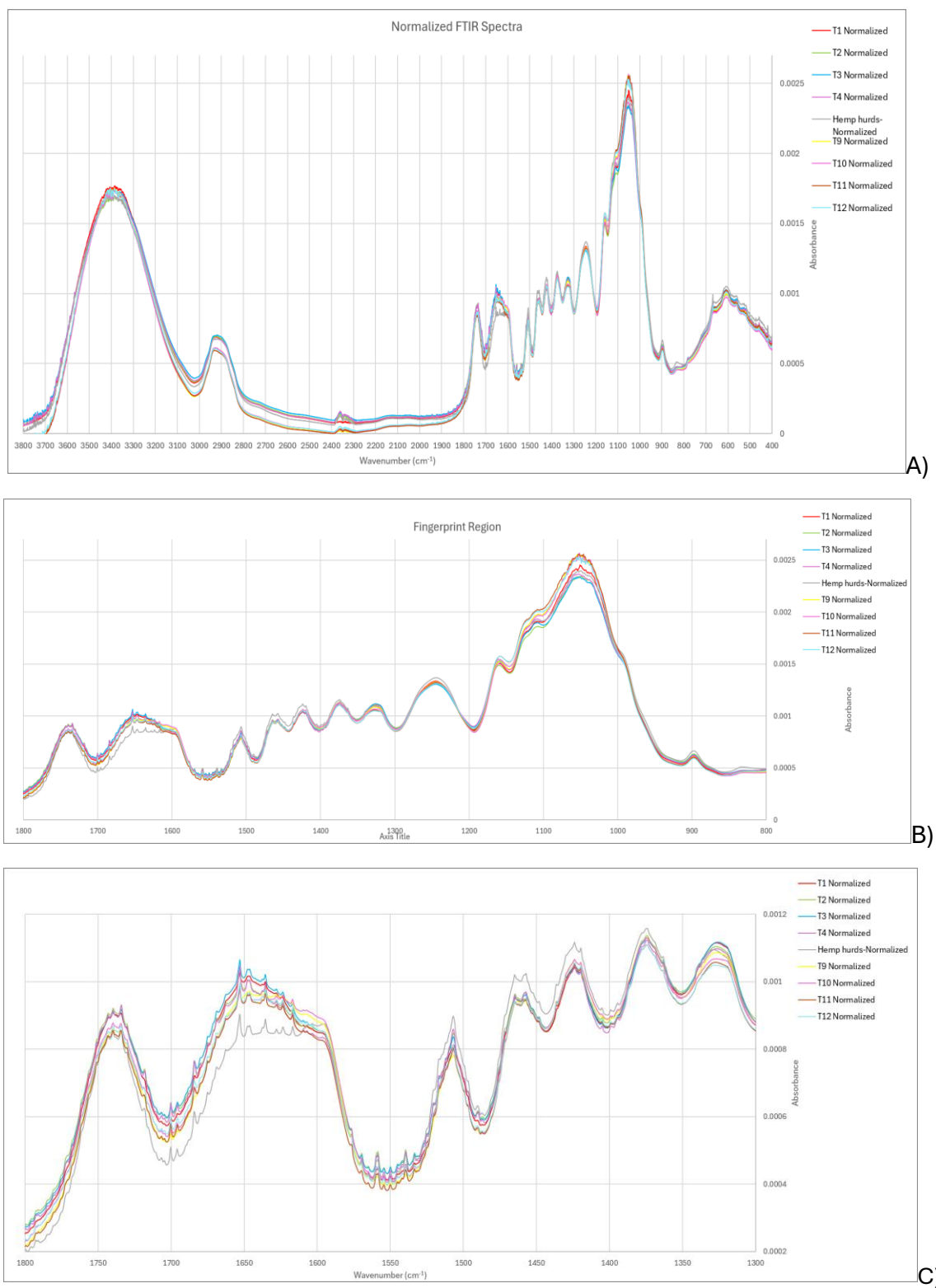


Figure 7. A) Complete normalized spectra of the 8 Treatments and hemp hurds control. B) Focused Fingerprint Region of the Spectra in the 800-1800 cm⁻¹ region. C) Focused region of the spectra in the 1300-1800 cm⁻¹ region.

Table 4. Functional group assignments to the observed peaks in FTIR spectra. Functional groups were assigned based on the standard FTIR library of functional groups in reference [22].

Wavenumber range cm^{-1}	Intensity/ Peak	Functional Group Assignment [22]	Compound Assignment
3550 - 3200	Strong Broad	O-H Stretching; Alcohol; Intermolecular bonded; N-H primary and secondary amine	Water, alcohols and phenols in cellulose and lignin [23]
3000-2850	2921 medium	C-H stretching; asymmetric; methyl, methylene, alkyl	Cellulose, hemicellulose, lignin, chitin, proteins, fungal lipids [23]
2365	Weak	O=C=O carbon dioxide	Atmospheric carbon dioxide
2320			
1762-1716	1744	C=O stretching; cyclopentanone or esters	Fungal lipids; metabolites
	1739	C=O nonconjugated stretching vibration	Fungal membrane or plant waxes Acetyl groups of hemicellulose [16]
	1731	C=O stretching; unsaturated ester or conjugated esters	Secondary metabolites or modified lignin or lipids and waxes
1700-1575	1700	C=O stretching; carboxylic acid or conjugated acid	Hemicellulose; Acidic fungal metabolites
Primary amide region	1694	C=O stretching	Fungal proteins or metabolites; chitin
	1685	C=O stretching; conjugated ketone	Aromatic ketones in fungal metabolites; proteins; chitin
	1681	C=O stretching; secondary amide or conjugated ketone	Fungal peptides; chitin

	1653	C=O stretching conjugated to -NH; Amide I band C=C stretching	Fungal proteins; chitosan; chitin [24]; lignin side face [25]
	1647	C=C stretching; conjugated alkene or aromatic ring	Lignin aromatic rings; fungal phenolics
	1635		
	1623	C=C stretching; aromatic skeletal vibration	Lignin; fungal pigments
	1616	C=C stretching; aromatic ring	Lignin; fungal phenolics
1540	weak	N-H bending; Amide II	Fungal proteins; Chitin [25]
1558	weak		
1525-1493	1507	C=C stretching in aromatic rings	Lignin [16]; pigments; phenolic compounds
1482-1445	1465	-CH ₃ bending; Scissoring and asymmetric deformation	Lignin; fungal cell walls
	1456	-CH ₃ asymmetric bending	Lignin; lipids; pectin methyl esters
1437-1406	1424	-CH ₂ scissoring bending	Cellulose; fungal glucans
	1420	OH bending/ CH ₂ deformation bending	Lignin; chitin
1390-1350	1373	CH ₃ bending; symmetric deformation	Lignin; pectin; lipids; chitin [26]
	1363	CH ₂ wagging; bending	Cellulose; fungal glucans; chitin
1350-1300	broad	C-N stretching; N-H bending	Chitin [27]; Fungal proteins; Cellulose & hemicellulose
	1310	C-O stretching	
1290-1193	Medium 1244	C-O stretching and bending	Cellulose
1193-1147	strong	C-O stretching, C-N stretching, N- H bending;	Cellulose; fungal proteins
1140-960	1112	C-O stretching;	Cellulose

	1048	C-O stretching and ring vibrations	Cellulose and hemicellulose [16]; pyranose rings of polysaccharides [28]
1025-1000	strong	Anomeric configuration of glycosidic linkages	Glucans [28]
900	medium	C-H bending; skeletal vibration; ring deformation	Cellulose; hemicellulose; glucans
830	weak	C-H bending; aromatic	Lignin; glucans [28]

Compounds were inferred from the functional groups, and where further literature recommendations were considered, citations have been provided in-column.

FTIR Semi-Quantitative Analysis Model

Previous studies characterizing plant materials attributed the integrated area under the curve between 1490 and 1530 cm^{-1} to lignin determination, as cellulose and hemicellulose do not have absorption bands in this region [29]. Here, we identified the same lignin band between 1493 and 1525 cm^{-1} with a peak at 1507 cm^{-1} which presents a higher absorbance in pure hemp hurds relative to the MBC samples. However, the melanin and phenolics produced by the fungal mycelium in the MBC samples may also contribute to the C=C stretching attributed to this band. Regardless, the area under the curve for the 1525-1493 cm^{-1} region has been assigned as the Lignin Index, as lignin's contribution is expected to be more significant than fungal metabolites.

Kostyukov et al. further assigned the area integrated between 1405 and 1480 cm^{-1} to cellulose [29]. Although the complex nature of our material splits this region into two peaks each with smaller peaks, the strong contribution of cellulose was observed in the notably higher absorption of hemp hurds relative to MBCs. In addition, the spectral peak at 1244 cm^{-1} (region 1290 - 1193 cm^{-1}) has been attributed to the C-O stretching and bending vibrations in the cellulosic backbone. Therefore, the sum of the area under these two regions was assigned as the Cellulose Index.

The region between 1762 and 1700 cm^{-1} exhibits absorbances associated with fungal lipids and metabolites, while also accounting for plant waxes. This region indicates a higher absorbance in MBC samples compared to pure hemp hurds, resulting from the mycelium-dependent

absorbances. Additionally, the neighboring region between 1700 and 1620 cm^{-1} region is primarily attributed to amide I, which is a predominant vibration in chitin, fungal proteins, and some metabolites [26, 27]. Since these two regions are attributed to compounds associated with the mycelium component of MBCs, the integrated area of this spectral region (1762 to 1620 cm^{-1}) was used as a proxy for relative mycelial contribution and was termed the Mycelium Index.

The Growth Index was developed as a semi-quantitative indicator of relative fungal colonization based on changes in mycelial and cellulose-associated spectral regions. Cellulose and mycelium composition of the MBCs are expected to change dependently. As cellulose is decomposed, it is expected that the mycelium in the sample increases. The ratio of mycelium index to cellulose index provides a metric that reflects how far this conversion has occurred, enabling a comparison between treatments.

Table 5. Mycelium, Cellulose, Lignin, and Growth Indices for each treatment condition and raw hemp hurds.

TREATMENT	MYCELIUM INDEX REGION (1762-1620)	CELLULOSE INDEX REGION (1290-1193 & 1482-1406)	LIGNIN INDEX REGION (1525-1493)	GROWTH INDEX
Hemp hurds	0.1008	0.1863	0.0244	0.0000
T1 -GL. SC. No Mg	0.1165	0.1800	0.0225	0.1062
T2 -GL. LC. No Mg	0.1172	0.1805	0.0232	0.1082
T3 -GL. SC. 2% Mg	0.1188	0.1795	0.0230	0.1207
T4 -GL. LC. 2% Mg	0.1163	0.1802	0.0231	0.1043
T9 -TP. SC. No Mg	0.1104	0.1799	0.0219	0.0727
T10 -TP. LC. No Mg	0.1114	0.1800	0.0220	0.0778
T11 -TP. SC. 2% Mg	0.1094	0.1787	0.0219	0.0711

T12 -TP. LC.				
2% Mg	0.1116	0.1793	0.0222	0.0812



Figure 8. Growth index of the eight treatments.

Three-way factorial ANOVA of the Growth Indices revealed that species was the only significant main factor ($p = 1.82E-5$), while $MgSO_4$ supplementation, growth duration, and all interactions between the factors were statistically not significant. Species identity, therefore, dominates the chemical growth response, with *G. lucidum* demonstrating an advantage of 45.4% higher Growth Index on average. Magnesium sulfate supplementation at 2% w/w caused a non-significant positive shift of 3.3%, demonstrating a favorable direction but within replicate noise at $n=3$.

For the comparative analysis of chitin, the area under the region resulting from C-N stretching and N-H bending vibrations ($1350 - 1300 \text{ cm}^{-1}$) was assigned as the Chitin Index [27]. This assignment was intended for relative comparison among treatments rather than absolute quantification because additional components of fungal and plant origin may contribute to absorbance within this region. Chitin also strongly absorbs in the $1694-1650 \text{ cm}^{-1}$ region, however, since this region has been included in the Mycelium Index, it has not been included in the Chitin Index. Compounds other than chitin, such as proteins, cellulose and hemicellulose, contribute to vibrations of the $1350-1300 \text{ cm}^{-1}$ region of the Chitin Index. The spectral overlap of cellulose and hemicellulose functional groups, which exist in high concentrations, dilutes the effect of chitin in

this region. Therefore, the Chitin Index should not be interpreted as a direct measure of chitin concentration. Instead, it functions as a comparative spectral metric whose biological interpretation relies on relative differences among treatments. To overcome this limitation, two ratio-based indices were calculated to illuminate chitin's contribution to the MBC chemical composition spectra.

To describe the relative amount of chitin in the entire biocomposite, the Composite Chitin Index was calculated. Since chitin content is a metric for mycelial growth [27], the Composite Chitin Index can reflect the mycelial density in the MBC. Additionally, the Mycelium Chitin Index was calculated as a value dependent on the Mycelium Index and Chitin Index, to produce a metric for the chitin content per unit of mycelium.

Table 6. Calculations of Composite Chitin Index and Mycelium Chitin Index.

TREATMENT	CHITIN INDEX REGION (1350 -1300)	COMPOSITE CHITIN INDEX	MYCELIUM CHITIN INDEX
Hemp hurds	0.0510	0.0000	0.0000
T1 -GL. SC. No Mg	0.0515	0.0123	0.2865
T2 -GL. LC. No Mg	0.0514	0.0109	0.3044
T3 -GL. SC. 2% Mg	0.0516	0.0140	0.3232
T4 -GL. LC. 2% Mg	0.0508	0.0085	0.3100
T9 -TP. SC. No Mg	0.0505	0.0070	0.2100
T10 -TP. LC. No Mg	0.0501	0.0048	0.2454
T11 -TP. SC. 2% Mg	0.0494	0.0029	0.2369

T12 -TP. LC.			
2% Mg	0.0492	0.0009	0.2899

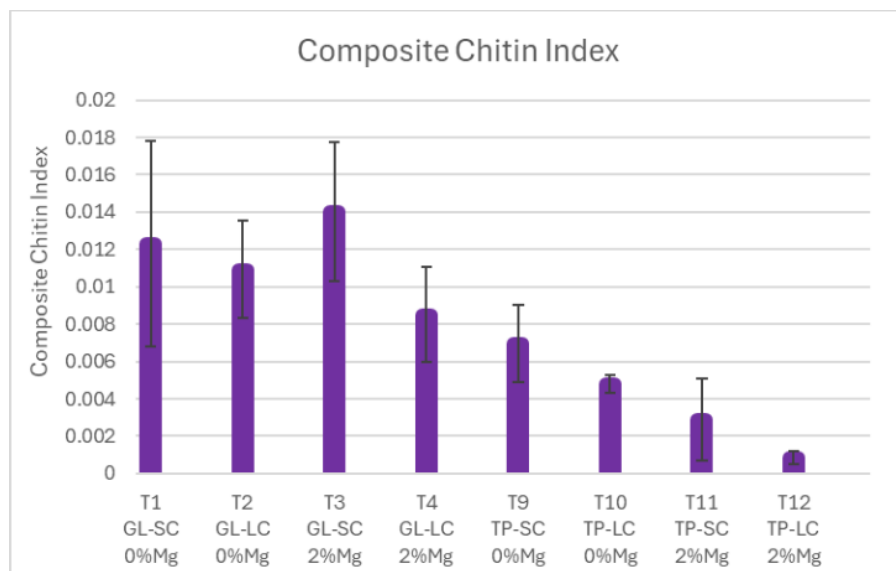


Figure 9. Composite Chitin Index of the eight treatments calculated based on formula 8.

For the Composite Chitin Index, both species ($p = 6.35E-6$) and growth duration ($p = 0.0499$) were significant three-way ANOVA factors, whereas $MgSO_4$ supplementation and all interaction terms were not significant. This suggests that the chitin-related signal measured at the whole-composite level was primarily determined by fungal species, with a weaker but statistically significant contribution from cultivation duration. Because the growth-duration effect was close to the $\alpha = 0.05$ threshold, this result should be interpreted cautiously, but it suggests that extending the substrate growth period reduced the relative chitin contribution within the composite matrix. Composite chitin index has been attributed to mycelial density, and findings suggest that longer growth reduces this density, at a borderline significant value. This aligns with the mechanical results, where growth duration significantly reduced compressive strength and stiffness, although the mechanical effect of duration was species-dependent. Furthermore, $MgSO_4$ supplementation falls just short of significance ($p = 0.0581$) in a negative direction, suggesting an inhibitory effect of Epsom salt supplementation at 2% w/w on mycelial density.

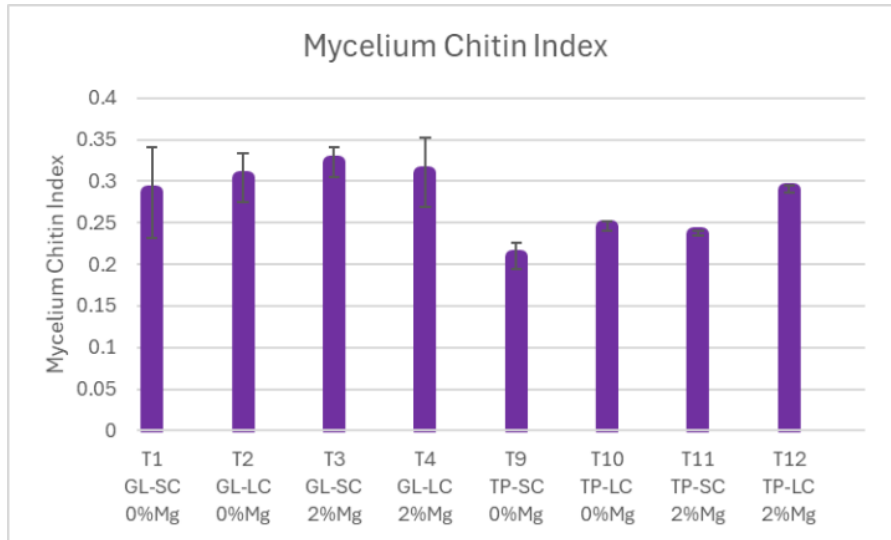


Figure 10. Mycelium Chitin Index of the eight treatments calculated according to formula 9.

For the Mycelium Chitin Index, species ($p = 1.05E-4$) and $MgSO_4$ supplementation ($p = 0.019$) were significant factors, while growth duration and all interaction terms were not significant. This indicates that the mycelium-associated chitin signal was influenced by both fungal species and $MgSO_4$ supplementation. Species is the dominant source of variation, with *G. lucidum* building more chitin-rich mycelia with an advantage of 23.8%. The Mycelium Chitin Index suggests that $MgSO_4$ may have specifically influenced fungal-wall-related chemistry. These results suggest that $MgSO_4$ -related improvements in mechanical performance may be associated with changes in mycelium-associated chitin chemistry. Furthermore, despite not being significant ($p = 0.076$), growth duration exhibits a clear trend where chitin accumulates in mycelium with age.

Physical Properties

Mycelial Skin

The outer layer of the MBCs comprises a layer of mycelium referred to as the mycelial skin. Since the hemp-mycelium substrate was mixed to complete homogenization prior to being shaped in formworks, and a fixed twenty-seven grams of mixed-substrate was molded, the mycelial skin and particle binding of the MBCs reflect growth during the nine-days of the composite cultivation period. The formation of the mycelial skin is clearly disturbed in magnesium-supplemented *T. pubescens* MBCs with this inhibition being more pronounced under long cultivation.

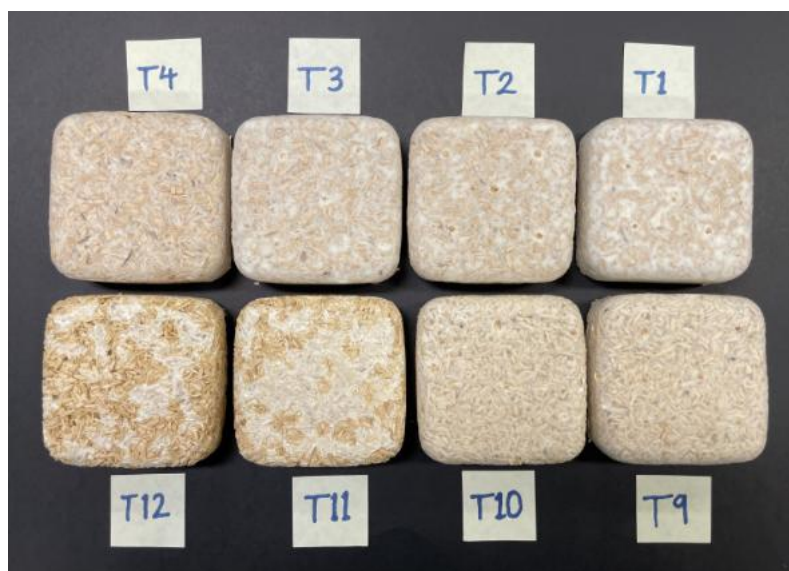


Figure 11. Visual comparison of the mycelial skin on the MBCs of different treatment conditions.

Density, Moisture Content, and Shrinkage

Table 7. Density, moisture content, and shrinkage of MBCs achieved under the eight treatment conditions.

Treatment	Density (kg/m ³)	Moisture Content (%)	Shrinkage (%)
T1 – GL, S.C. No MgSO ₄	118.1 +/- 1.6	47.6 +/- 0.9	10.0 +/- 0.7
T2 – GL, L.C. No MgSO ₄	111.5 +/- 2.5	50.6 +/- 0.5	9.3 +/- 0.9
T3 – GL, S.C., 2% MgSO ₄	117.9 +/- 2.3	49.0 +/- 0.5	8.2 +/- 1.3
T4 – GL, L.C., 2% MgSO ₄	121.2 +/- 1.3	45.4 +/- 1.1	7.9 +/- 0.5
T9 – TP, S.C. No MgSO ₄	103.2 +/- 1.5	49.5 +/- 1.2	5.9 +/- 1.0
T10 – TP, L.C. No MgSO ₄	102.3 +/- 0.9	54.0 +/- 0.3	7.7 +/- 1.6
T11 – TP, S.C., 2%MgSO ₄	107.5 +/- 1.4	51.4 +/- 0.4	7.0 +/- 1.1
T12 – TP, L.C., 2%MgSO ₄	104.2 +/- 1.6	53.2 +/- 0.6	7.2 +/- 1.2

Three-way ANOVA of density results confirmed a significant effect for the three main effects of species ($p = 2.71\text{E-}47$), cultivation duration ($p = 3.28\text{E-}7$), and MgSO₄ supplementation ($p = 1.45\text{E-}16$). Additionally, the two-way interaction between cultivation duration x Epsom salt

supplementation ($p = 1.78\text{E-}6$), and the three-way interaction between all three effects ($p = 7.18\text{E-}13$) were significant.

Comparison of the density of the treatments supports the importance of the choice of organism, as the *T. pubescens*-MBCs significantly produce a lower-density material than *G. lucidum* MBCs. For GL, 2% MgSO_4 supplementation is harmless under short cultivation, but inflicts the largest density penalty under long cultivation. In TP, Epsom salt raises density at both growth durations – more under short cultivation than long. Both organisms delivered the lowest density material through the 20-day matrix cultivation without MgSO_4 supplementation (T2 and T10), in affirmation of the hypothesis that longer growth reduces density. Furthermore, these two low density treatments held the highest moisture content and the lowest Young's moduli relative to treatments of the same species.

Hydrodynamic Characterization

Table 8. Water absorption rate of treatments after 1 hour, 4 hours, and 24 hours of immersion in deionized water.

Treatment	Water Absorption Rate after 1h immersion (%)	Water Absorption Rate after 4h immersion (%)	Water Absorption Rate after 24h immersion (%)
T1 – GL, S.C., No MgSO_4	14.81 +/- 1.0	36.02 +/- 8.4	90.76 +/- 16.3
T2 – GL, L.C., No MgSO_4	21.94 +/- 1.6	51.51 +/- 7.1	122.31 +/- 8.8
T3 – GL, S.C., 2% MgSO_4	16.80 +/- 1.2	34.93 +/- 6.4	84.89 +/- 12.2
T4 – GL, L.C., 2% MgSO_4	26.52 +/- 5.8	75.61 +/- 11.9	149.95 +/- 12.2
T9 – TP, S.C., No MgSO_4	121.37 +/- 66.3	306.73 +/- 39.6	398.38 +/- 30.2
T10 – TP, L.C., No MgSO_4	110.97 +/- 22.5	238.42 +/- 30.2	343.83 +/- 25.2

T11 – TP, S.C., 2% MgSO₄	42.34 +/- 8.2	126.91 +/- 54.4	225.68 +/- 77.8
T12 – TP, L.C., 2% MgSO₄	55.74 +/- 2.5	158.28 +/- 52.2	248.87 +/- 54.3

TP-MBCs hold a much higher WAR when compared to GL-MBCs, under every fabrication condition. Due to the strong effect of species which confounded the three-way ANOVA between treatments, two-way ANOVA was conducted for each species independently for 24h WAR results, with cultivation duration and MgSO₄ supplementation as the main effects. Results revealed that for GL-MBCs, the effect of growth duration had a significant effect ($p = 1.68\text{E-}4$), while Epsom salt supplementation did not have a significant effect. Conversely, for TP-MBCs, Epsom salt supplementation had a significant effect ($p = 1.96\text{E-}3$), while cultivation duration did not have a significant effect. No significant interaction between the two variables was observed in either species. Findings illustrate an important engineering trade-off; conditions that favor lightweight materials may also increase moisture sensitivity.

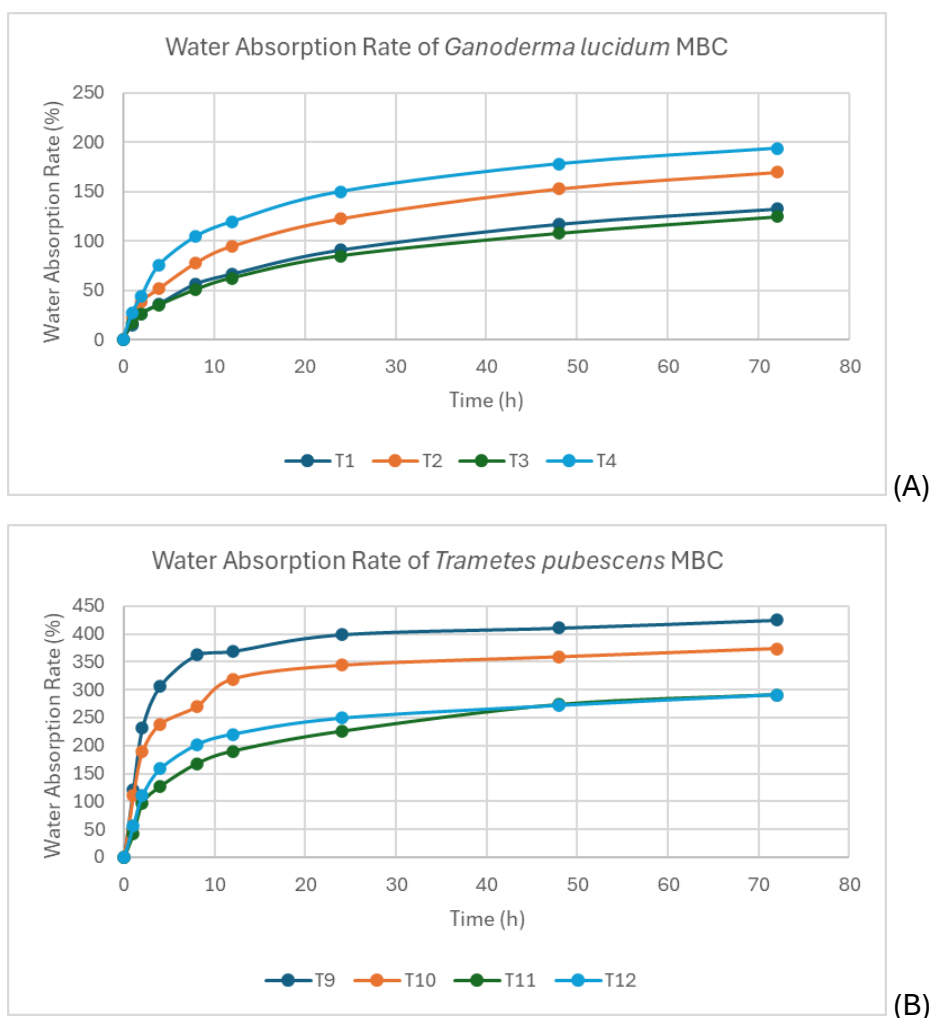


Figure 12. Water absorption rates of (A) *G. lucidum* MBCs and (B) *T. pubescens* MBCs for the 72-hour investigation period.

During the water absorption tests, the expression of pigmented metabolites (melanin or its derivatives, or carotenoids) in the 20-day cultivation treatments was observed through a change in the color of the deionized water after 24 hours of immersion. Figure 13 captures such dissolved pigmented metabolites in the water which are present in both organisms, despite this effect being less evident for *G. lucidum*. This change in the color of the water after 24 hours of sample immersion suggests that metabolites in the MBCs – possibly a type of melanin, dissolve in the water over time. When attributing the intensity of the yellow color to estimated concentration of this colored metabolite, the 20-day substrate cultivation treatments of *T. pubescens* (T10 and T12) not only present strong evidence of relatively higher concentrations of these metabolites but further describe an even higher expression when supplemented with MgSO_4 in T12.

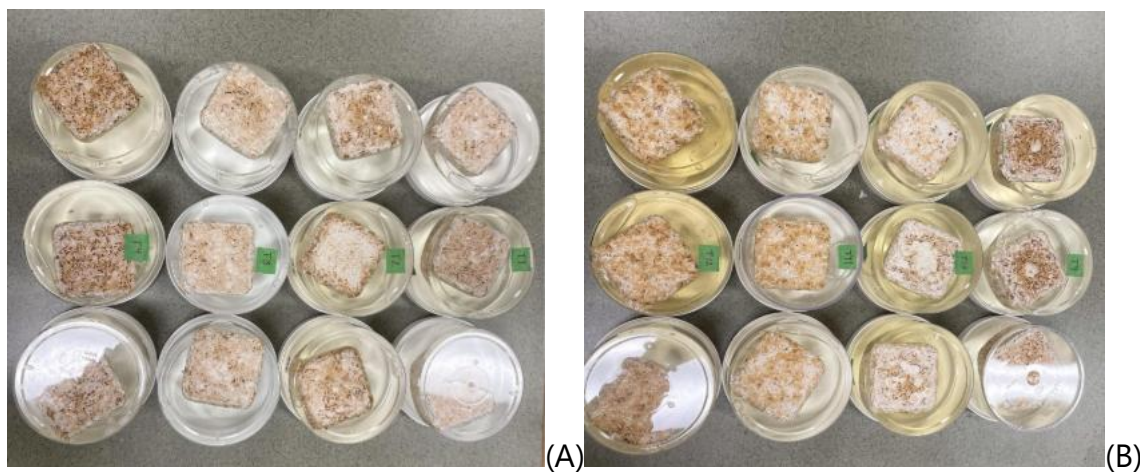


Figure 13. Evidence of soluble pigmented metabolites produced during cultivation, observed after 24h of immersion. Triplicates of each treatment are vertically positioned, middle sample labels treatment ID. (A) *G. lucidum* T1-4 (B) *T. pubescens* T9-11

Discussion

The results demonstrate that biological process variables can be leveraged as engineering design parameters for tailoring MBC performance. Among the investigated factors, fungal species exerted the strongest influence on mechanical, physical, and chemical properties. MgSO_4 supplementation emerged as a secondary control variable capable of modifying both performance and cell-wall associated chemistry, while cultivation duration influenced the balance between density and mechanical performance. The highest compressive strength of 73.23 kPa and Young's modulus of 17.75 kPa were achieved by *G. lucidum* under short cultivation with 2% w/w MgSO_4 supplementation. Thermal conductivity changed minimally among treatments, between 0.050 and 0.055 $\text{kW}\cdot\text{m}^{-1}\cdot\text{K}^{-1}$. Density was tunable in the 102.3 to 121.2 kg/m^3 range. The WAR proved to be highly organism dependent with *G. lucidum* having a much lower water absorption rate than *T. pubescens*. The FTIR-derived Mycelium Chitin Index increases under MgSO_4 supplementation in *T. pubescens*, in support of our hypothesis for the supportive role of the Mg^{2+} cation as a cofactor to chitin synthase (ChS), although this effect was less evident in *G. lucidum* MBCs, indicating a different ChS profile between the species.

Each stage of the fabrication strategy described herein was designed with bioprocess scaling considerations and sustainability as priorities. Firstly, the hemp hurds were not initially soaked in water for hydration, opposing literature suggestions on soaking. It was presumed that the high pressure and temperature of autoclaving the substrate with the water volume required for 85%

w/w moisture content in sealed autoclavable bags was sufficient for achieving sufficient substrate hydration. The final moisture content of the MBC samples ranged between 45.4-54.0%, in support of the hydration sufficiency of the fabrication conditions. Secondly, spawn preparation often involves food-grade grains such as Sorghum or Cereal grain [30,31]. However, we used the same hemp hurds source as the substrate phase for spawn production under the same supplementation routine. This, along with the introduction of hemp hurds in the inoculum media, were done to activate the fungal metabolic pathways responsible for effective hemp hurds colonization early during growth. Furthermore, the replacement of food-grade grains with hemp hurds industrial byproduct reduces the operational cost of the bioprocess and improves its sustainability. Thirdly, investigations of the effect of mixing confirmed that pre-culture cultivation prior to molding is optimal for MBC fabrication, which was also the approach taken here [25]. However, the effect of mixing the incubated substrate every 48-72 hours throughout the cultivation period, as done here, requires further investigation.

Previous investigations of supplementation with magnesium salts were targeted at the uptake of the cation by fungi for nutritional purposes [32]. Sawdust medium was supplemented with 0.5%, 1%, and 5% of different magnesium salts. Among the salts, MgSO_4 presented mild growth inhibition on *Hypsizygus marmoreus*. This supplementation was most ideal at 0.5% and was not analyzed in relation to chitin expression [32]. The investigation of the effects of MgSO_4 supplementation herein began with radial growth measurements on PDA + 2% w/w hemp hurds inoculum plates supplemented with and without 0.4% w/w MgSO_4 , which did not detect a difference in growth rate under supplementation at this concentration. The next stages of spawn and substrate cultivation were supplemented with 2% w/w MgSO_4 to ensure the effect of the cation is pronounced. Additionally, it is expected that the acclimatization of the species towards the supplement through the slow introduction of MgSO_4 in the inoculum stage (at 0.4%) activates metabolic pathways that can facilitate the mycelium's tolerance of the Epsom salt. Broadly, results justify relating 2% w/w MgSO_4 supplementation to an increase in strength, stiffness, density, and Mycelium Chitin Index on an organism-dependent and growth-time specific basis.

Previous investigations of the effects of growth time are conflicting in that both positive and negative impacts of extended cultivation time on MBC properties have been reported. While Ghazvinian et al. found that compressive strength decreases when total cultivation time increases, the results of Song et al. demonstrated differing effects for growth time depending on the species

[15, 16]. The latter has been confirmed here, where the results report that while increased substrate growth time reduces compressive strength in GL-MBCs, this behavior is not consistent with TP-MBCs.

Both fungal species studied possess trimitic hyphal networks. However, imaging described *G. lucidum*'s hyphal network to be mostly constructed of unbranched skeletal hypha, unlike *T. pubescens* highly branched hyphal network of generative/ligative hypha. Theoretical modeling of the effect of each hyphal type has revealed that skeletal hypha are most influential on the mechanical properties of fungal sporocarps [33]. Such morphological differences between the organisms, in addition to the potential effect of biochemical and metabolic differences, combined with the higher mycelial growth rate of *G. lucidum*, resulted in themes in the properties of GL-MBCs relative to TP-MBCs. Generally, GL-treatments deliver a material with lower WAR, higher density, higher Chitin Indices and Growth Index, and higher compressive strength and Young's modulus.

Compressive strength and Young's modulus of the MBCs are used to calculate the Specific Strength Ratio, Strength-to-Weight ratio, and Specific Modulus, which are critical in considering the material for applications in the civil engineering industry [34]. Both species delivered MBCs with the highest compressive strength and Young's modulus under MgSO_4 supplemented short cultivation (T3 and T11). Since Mg^{2+} is a cofactor for the ChS enzyme responsible for chitin synthesis in the mycelium, this significant increase in strength in these supplemented treatments could be related to an increase in the chitin content of the mycelium [12]. The Mycelium Chitin Index of these two treatments is also on average higher than the short cultivation without MgSO_4 treatment, indicating a correlation between strength and Mg^{2+} availability. Although increased Mycelium Chitin Index values coincided with improvements in compressive strength and stiffness, the present study does not establish a causal relationship between chitin content and mechanical behavior. Simultaneous changes in density, hyphal architecture, substrate degradation, and other fungal-wall constituents may also contribute to the observed responses.

Generally, a slight increase in Young's modulus can be observed in every condition when MgSO_4 supplementation is provided. This increase in stiffness further suggests an increase in chitin expression via MgSO_4 supplementation, as chitin is a high strength natural polymer. Transmission Electron Microscopy of trimitic fungi indicated that the inner rigid portion of the fungal-wall, which is composed of β -glucans and chitin accounts for rigidity of the fungal cell, emphasizing the role of chitin in material strength [6]. The FTIR-based Mycelium Chitin Index also increased when MgSO_4

was added to the substrate, further supporting a correlation between Young's modulus and chitin expression in the mycelium. The increase in Mycelium Chitin Index under supplementation, specifically in *T. pubescens*, provides indirect support for the proposed role of magnesium in chitin biosynthesis. This relationship between material strength and chitin expression opens doors for future investigations regarding tunable material strength.

A previous investigation of the thermal conductivity of MBCs reported that the composites produced from hemp presented a lower thermal conductivity ($0.0404 \text{ W.m}^{-1}.\text{K}^{-1}$) when compared to straw and flax-based MBCs, indicating dependency of thermal conductivity on the substrate [7]. The thermal conductivity of the hemp hurds MBCs produced herein ranged between 0.0495 to $0.055 \text{ W.m}^{-1}.\text{K}^{-1}$. The lowest thermal conductivity resulted from production with *T. pubescens* under long cultivation in the absence of Epsom salt supplementation – the same treatment that yielded the lowest density material. The narrow tunability range for thermal conductivity across the treatments further supports the dependency of this parameter on the substrate's nature rather than other production conditions.

The combination of the lignocellulosic hemp hurds and the mycelium tissue provide a basis for the anticipated chemical composition of the MBC samples. Plant wastes such as hemp hurds are largely composed of cellulose, in addition to hemicellulose, pectins, lignin, and other minor components, including bound water, proteins, waxes, pigments, and inorganic materials [35]. The fungal mycelium on the other hand is rich in lipids, proteins, glucans, and chitin. The FTIR spectra therefore reflect the vibrations of the collective of these compounds. Characterization of materials through the analysis of the relative proportions of compounds based on FTIR spectra has been previously reported [35, 36]. Since both species are white-rot fungi, they degrade both the lignin and cellulose in the hemp hurds, yet the exact ratio of these compounds remaining in the final MBC is unique to each organism. Previous reports indicate that the strength and durability of the composite is a product of lignin composition, while water absorption and degradability are determined by carbohydrate content [34]. Here, MgSO_4 supplemented short cultivation treatments in both species (T3 and T11) held the lowest Cellulose Index and WAR across treatments of their own species batch. Moreover, T3 and T11 hold the highest compressive strengths, but no correlation with the Lignin Index was found.

From the integrated area of various regions of the FTIR spectra, the Growth Index was derived from a ratio of the Mycelium Index to Cellulose Index. The region assigned to Mycelium

Index presents a limitation in that in addition to representing components of the fungal mycelium (lipids, peptides, metabolites), it is inclusive of vibrations of hemp components such as plant waxes, proteins, and lignin. Similarly, the Cellulose Index aggregates bands that are not exclusive to cellulose, confounding the Index by lignin, pectin, and fungal lipids. Regardless, to calculate the Growth Index, the ratio of the two indices for each sample treatment has been subtracted from that of pure hemp hurds as the reference spectrum. The subtraction is founded on the assumption that when the ratios for the reference hemp hurds spectrum are subtracted from the ratios of the sample spectra, the derived indices reflect the contribution of the mycelium to each spectrum [37]. Hence, the Growth Index reflects the mycelium's concurrent role in increasing absorbance in the Mycelium Index region, and reducing absorbance in the Cellulose Index region, both being a product of the mycelium's growth. Since the wavenumber range selected for the Mycelium Index was inclusive of absorbances resulting from metabolites, it is possible that T11 and T12 produced higher metabolites content to maintain their relatively high Growth Index, despite having a lower total MBC mycelial density as per their lower Composite Chitin Index. *G. lucidum* treatments generally hold a higher Growth Index relative to *T. pubescens*, consistent with the higher growth rate of *G. lucidum*.

Since the total amount of chitin in the sample indicates the amount of mycelium, the Composite Chitin Index provides a relative comparison of the mycelium density in the MBC samples analyzed [20]. The highest Composite Chitin Index was calculated for the GL-SC-2%Mg treatment (T3), which also exhibited the lowest WAR and highest compressive strength across all conditions. This measure of mycelium density in the composites is aligned with visual images in Figure 11, where T11 and T12 hold the least amount of mycelium on the composite skin. As expected, the higher growth rate of *G. lucidum* results in a higher Composite Chitin Index and overall mycelial density.

The comparison of the Mycelium Chitin Index suggests that in *T. pubescens*, chitin per unit of mycelium increases under supplementation with MgSO_4 in both growth-time routines. This is consistent with the hypothesis that the availability of the Mg^{2+} cation as the divalent metal cofactor to the enzyme ChS would increase chitin content in the mycelium. ChS enzymes in fungi have been divided into seven groups and phylogenetically clustered into three clades, with the same type of ChS in different fungi showing high similarity [38]. The function of each ChS belongs to specific biological processes in the fungus, such as hyphal tip expansion, sporulation, and cell wall stability. *G. lucidum*'s ChS (GlChS) profile has been bioinformatically studied to have eight ChS

family members which were phylogenetically divided into five classes, with diversity in their physicochemical properties [39]. The Mycelium Chitin Index of GL-treatments does not indicate a clear increase in mycelial chitin when fabrication is supplemented with MgSO_4 , suggesting that the GlChS responsible for cell wall stability possibly uses a different divalent ion as its cofactor, or that the regulation of chitin synthesis in *G. lucidum* is not influenced by the availability of MgSO_4 . On the other hand, *T. pubescens* presents a clear increase in Mycelium Chitin Index when supplemented with MgSO_4 , with this measure of chitin per unit of mycelium being higher under long-cultivation fabrication routines. Additionally, the observation of increased metabolite production in T10 and T12 in Figure 13 supports the increased Mycelium Chitin Index, as some metabolites such as solubilized melanin often covalently link to chitin in the cell wall, protecting it from chitinase activity [40]. These results suggest that Mg^{2+} availability may influence chitin production differently in *T. pubescens* and *G. lucidum*. Interestingly, these supplemented TP treatments with higher chitin content per unit of mycelium possess a lower WAR than the equivalent treatments without supplementation, suggesting an increased hydrophobic effect in the MBCs because of the higher chitin content of the mycelium, despite their disrupted mycelial skin.

The evaluation of the physical properties of the MBCs is critical for determining the fabrication routine that yields the best material for application in packaging. Hemp hurds fabrication with *T. pubescens* under 20-day substrate cultivation without any supplementation yields a material with the lowest density of $102.3 \pm 0.9 \text{ kg/m}^3$, with an intact mycelial skin around the material that prevents particle dispersion. However, the equivalent short-cultivation treatment (TP-SC-0%Mg) is only 1.07 Kg/m^3 heavier, which makes the two statistically indistinguishable, yet allowing saving the extra production time. Moreover, the high WAR of these MBC treatments provides an advantage for the shipment of liquid products that may be subject to leaks or spills during transport. However, for packaging of electronics products, the low WAR of GL-SC-2%Mg treatment and its subsequent high compressive strength is more suitable, despite its higher density trade-off.

The higher growth rate of *G. lucidum*, which brings about a denser mycelial network, potentially increases pore connectivity while limiting pore volume, resulting in a reduced water absorption [21]. Contrarily, the lower growth rate of *T. pubescens* preserves a more porous MBC structure that leads to localized saturation and a higher water absorption rate (WAR). Motamedi et al. assigned the sorption in MBCs to spatial configuration and connectivity of the hyphal network,

after total porosity and fungal species [21]. Such structural considerations could explain the reason behind a much higher water uptake by the TP-0%MgSO₄ compared to the supplemented *T. pubescens* samples. The outer mycelial skin in T9 and T10 unsupplemented treatments creates a closed internal structure where water entering during immersion gets trapped, unlike T11 and T12 supplemented treatments where the incomplete mycelial skin allows the movement of water in and out of the structure. Additionally, the Mycelium Chitin Index of T11 and T12 supplemented treatments is higher than unsupplemented treatments, indicating that despite a relatively lower amount of mycelium in these MBCs (as per the lower Composite Chitin Index), the chitin content of the existing mycelium is higher. The hydrophobic behavior of chitin in T11 and T12 may be further contributing to the lower WAR of these treatments. When comparing the WAR of GL-MBCs, long cultivation treatments hold a higher WAR than short cultivation treatments, supporting the hypothesized higher porosity under long cultivation. Generally, cultivation conditions of short cultivation and 2% MgSO₄ supplementation produced the MBC with the least WAR in both species. In a previous study using *Ganoderma* species where secondary demolded cultivation was implemented to produce intact mycelial skin, the WAR of MBCs after 1 hour of immersion was found to be equal to (25%) or greater than (42%) the WAR observed here by *G. lucidum* [25]. This indicates that the micro-structure of the MBC and the mycelium properties primarily drive the water resistance of the material, and that the mycelial skin exhibited a secondary effect.

The GL-20-days matrix cultivation in supplementation with MgSO₄ (T4) presented a notable increase in the WAR of the material after 24 hours of immersion compared to long-cultivation without Epsom salt supplementation (T2). This suggests that during long cultivation, MgSO₄ supplementation reduces the hydrophobic mycelial density, consistent with the lower Composite Chitin Index and higher density of T4 relative to T2. Regardless of this speculated lower mycelial density of T4 compared to T2, in light of the effects of Epsom salt, T4 exhibited a significantly higher compressive strength than T2. While a preliminary conclusion would suggest that lower mycelial density is correlated with higher strength, closer inspection of T3 negates this hypothesis. T3 presented the highest Composite Chitin Index and mycelial density, and compressive strength, contradicting the hypothesis of more mycelium reducing strength. The simultaneous consideration of T4's Composite Chitin Index, Mycelium Chitin Index, density, and WAR depict a material with lower relative mycelium yet high chitin in its mycelium, producing the unique material properties of T4.

Limitations of this research are multifaceted. FTIR bands in mixed materials overlap for various compositional elements, which prevents accurate quantification. Pure chitin was not subject to FTIR analysis as a reference, and its wavenumber assignment was through inferring functional groups and literature recommendations. The developed FTIR indices are semi-quantitative, requiring supplementary chemical analysis for accurate quantification of MBC chemical composition. The study is further limited to the effects of three biological parameters, lacking holistic optimization of the bioprocess. Considering the inherent variability of living organisms, the three biological replicates in mechanical tests and the duplicates in thermal conductivity measurements are a limitation in dataset scope, reducing the strength of the conclusions. Lastly, the effect of magnesium sulfate has been studied at a single concentration of 2% w/w. Although this single-dose experiment sets ground as the first instance of the study of MgSO_4 as an MBC supplement, future MBC optimization studies with a range of concentrations are required due to the study's limitation in investigating a dose-response relationship.

Conclusion

To conclude, the properties of hemp hurds-based MBCs are considerably tunable through the strategic selection of substrate growth time, MgSO_4 supplementation regime, and species. The lowest density material was obtained through the *T. pubescens* 20-days non-supplemented protocol which held a density of $102.3 \pm 0.9 \text{ kg/m}^3$. The material exhibits partial viscoelasticity, with up to 55% deformation recovery after one hour of load removal depending on the treatment. The lowest water absorption rate (WAR) during the first 4-hours of immersion was for the *G. lucidum* 12-days cultivation with 2% MgSO_4 supplemented material with its WAR at $34.93 \pm 6.4\%$. The same treatment condition produced a material with the highest compressive strength at $73.23 \pm 13.5 \text{ kPa}$ at 10% strain. The highest compressive strength across the species batches belonged to 12-days cultivated MgSO_4 supplemented treatments. MgSO_4 supplementation significantly improved mechanical properties under the conditions investigated. Young's modulus reduces under longer cultivation periods and increases under MgSO_4 supplementation. Thermal conductivity ranged between 0.0495 and $0.055 \text{ W}\cdot\text{m}^{-1}\cdot\text{K}^{-1}$. The FTIR spectroscopy analyses suggest an increase in mycelial chitin in *T. pubescens* following supplementation with MgSO_4 , despite possible overall growth inhibition. The findings demonstrate that fabrication conditions can be strategically tailored to target specific end-use requirements.

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Conflict of Interest

The authors declare no conflict of interest.

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Chapter 3: Conclusions and Engineering Significance

Mycelium-based composites (MBCs) have emerged as novel alternatives to synthetic foams and wood products. To achieve their industrial adoption, controlled tuning of their properties is required for specific applications. In addition to the choice of substrate and species, cultivation conditions play a critical role in determining the properties of MBCs. The effects of supplementation with magnesium sulfate and variation of growth duration were investigated as control levers for the tunability of MBC properties. Between *Ganoderma lucidum* and *Trametes pubescens*, *G. lucidum* is capable of producing a material with higher stiffness, while compressive strength suggests higher dependency on production conditions. Findings position magnesium sulfate as a useful supplement for increasing the compressive strength and Young's modulus of the material, with species-specific sensitivity to the supplement. Chemical composition analysis suggests that increased chitin in the mycelium due to supplementation may be responsible for improved strength under supplementation routine. Although longer growth durations can be leveraged for a slight decrease in density, a trade-off between longer growth and process life cycle must be considered. The role of growth time is organism-dependent; however, a common trend that was observed suggests 12-days of substrate growth yielding a stronger material and 20-days of substrate growth yielding lower density.

Knowledge of the effect of various conditions on the material characteristics enables property-driven design of MBCs. This work brings forward the intentional tuning of mechanical properties through controlled biological parameters, contributing to the transition from empirical to design-based engineering. The relationship between magnesium sulfate supplementation and chitin content not only explains the increased mechanical strength of the material but also expands the findings on the role of magnesium beyond the MBC industry and to the fungal chitin production industry, strengthening the engineering significance of the findings. FTIR indices described here may serve as non-destructive predictive tools, enabling mechanical performance prediction, quality control, and material classification. Findings contribute to process standardization with the intention of batch-to-batch consistency and scale-up feasibility.

Future work may expand the optimization of the effect of magnesium sulfate supplement by evaluating a range of concentrations, as this work was founded on the study of a single concentration of the supplement. Further refinement of the FTIR indices can be achieved through

peak deconvolution rather than integrated spectral regions. FTIR-derived indices may be validated against independent pure chemical measurements for reliability. Additionally, due to the limitation of spectral overlap in FTIR analyses, complementary chemical characterizations such as solid-state NMR can strengthen understanding of the chemical origins of mechanical behavior. Since a relationship between chemistry and mechanics has been established herein, future work has a foundation for developing predictive structure-property models. Such models could ultimately allow engineers to estimate material performance from rapid FTIR tests without destructive methodology. The necessity for continued investigation of nutritional, environmental, and biological parameters persists for protocol standardization. Long-term durability studies such as moisture aging, freeze-thaw cycles, UV exposure, etc. hold engineering significance for real-world applications. Since reproducibility of laboratory-scale results during industrial production remains in question, scale-up studies are an inevitable avenue of future work.