Aalto University
School of Chemical
Engineering

Lauri Peuhkurinen

FUNGAL MYCELIUM AS A MATERIAL COMPONENT: PRODUCTION AND CHARACTERIZATION OF GENETICALLY ENGINEERED STRAINS

Master´s Programme in Life Science Technologies
Major in Biosystems and Biomaterials Engineering

Master's thesis for the degree of Master of Science in Technology
submitted for inspection, Espoo, 21st of November, 2022.

Supervisor	Professor Markus Linder
Instructor	Géza Szilvay, Ph.D.

Aalto University
School of Chemical
Engineering

Aalto University, P.O. BOX 11000, 00076 AALTO
www.aalto.fi

Abstract of master's thesis

Author Lauri Peuhkurinen

Title of thesis Fungal mycelium as a material component: production and characterization of genetically engineered strains

Degree Programme Master's programme in Life Science Technologies

Major Biosystems and Biomaterials Engineering

Thesis supervisor Professor Markus Linder

Thesis advisor Géza Szilvay, Ph.D.

Date 14.11.2022 **Number of pages** 79 **Language** English

Abstract

Mycelium has potential in fulfilling the sustainable materials needs of the future by providing an alternative for various traditional material solutions, such as animal leather and construction materials. Mycelium is produced by fungi and is inherently bio-based. Fungal materials are typically mycelium materials produced either from cultured fungi or fruiting bodies of fungi. Mycelium is the vegetative part of fungi made of branching hyphae composed of fungal cells. Cell walls are an important structural part of fungal cells. Thus, it is likely that cell wall regulation pathways affect the microstructure of the fungal cells and ultimately the material properties of mycelium.

In this work, fourteen *Trichoderma reesei* strains were studied to see how the genetic engineering of single genes affected material properties of mycelium. The mycelium film production process was based on the previously patented method. A shake-flask fermentation was used to cultivate mycelium biomass that was then treated to obtain mycelium suspension that was then cast as a mycelium film. Yield, microstructure and protein expression patterns of mycelium culture, cell wall polysaccharide content of mycelium biomass, and tensile strength, strain and Young's modulus of mycelium films were determined. These values were compared to the genetic differences of the studied fungal strains.

This work provided a look into the production of mycelium films and the investigation of the material properties with comprehensive analysis methods. The cell wall polysaccharide content analysis was further developed with the experiments of this work. The mycelium microstructural differences were generally minor between samples and their references. Additionally, the hyphae of the wild-type (Rut-C30 and QM9414) and directly wild-type-derived fungal strains were longer and only mildly branching than the more genetically engineered strains and their transformants. The results suggested that genetic engineering reduced the polysaccharide content of the cell wall of the studied strains. The results for physical and mechanical properties were less consistent and both reducing and increasing effects were observed. This work provides a basis for further development of novel mycelium materials. The future of mycelium materials is indeed filled with lots of potential.

Keywords mycelium, filamentous fungi, hyphae, cell wall integrity signalling pathway, Trichoderma reesei, chitin, glucan, galactomannan, polysaccharide, cell wall composition, cell wall stress, biosynthesis, biofabrication, hydrophobin, resilin, silk, chitinase, physical and mechanical properties, density, tensile strength, strain, Young's modulus, protein production, gene expression, RUT-C30, QM9414

Aalto-yliopisto
Kemian tekniikan korkeakoulu

Aalto-yliopisto, PL 11000, 00076 AALTO
www.aalto.fi
Diplomityön tiivistelmä

Tekijä	Lauri Peuhkurinen
Työn nimi	Sienirihmasto materiaalien rakenneosana: geenimuokattujen sienikantojen tuotanto ja karakterisointi
Koulutusohjelma	Life Science Technologies -maisteriohjelma
Pääaine	Biosystems and Biomaterials Engineering
Työn valvoja	Professori Markus Linder
Työn ohjaaja	FT Géza Szilvay
Päivämäärä 14.11.2022	**Sivumäärä** 79 **Kieli** Englanti

Tiivistelmä

Myseelillä, eli sienirihmastolla, on hyvät edellytykset täyttää kestävän materiaalin tunnusmerkit tulevaisuudessa tarjoamalla vaihtoehdon perinteisille materiaaliratkaisuille, kuten eläinnahkalle sekä erilaisille rakennusmateriaaleille. Myseeli on sienten tuottama luontaisesti biopohjainen materiaali. Sienimateriaalit ovat tyyppillisesti myseelimateriaaleja, jotka tuotetaan joko sieniviljelmillä tai sienten itiöemistä. Myseeli koostuu sienisoluista rakentuvista sienirihmoista, joka muodostaa sienen kasvavan osan. Soluseinillä on rakenteellinen merkitys sienisoluissa. Onkin todennäköistä, että soluseinän sääntely ja viestinvälitysketjut vaikuttavat sienisolujen mikrorakenteeseen ja kaiken kaikkiaan myseelin materiaalio minaisuuksiin.

Tässä työssä käytettiin neljäätoista *Trichoderma reesei* -kantaa arvioimaan geneettisten muokkausten vaikutusta myseelin materiaalio minaisuuksiin. Myseelikalvon tuotantoprosessi pohjautui aiemmin patentoituun menetelmään. Myseelibiomassaa valmistettiin pullokasvatusfermentoinnilla, ja tämä biomassa käsiteltiin suspensioksi, joka valettiin myseelikalvoksi. Myseelikasvatuksen saanto, mikrorakenne, tiettyjen proteiinien tuottotasot sekä myseelibiomassan soluseinän polysakkaridipitoisuus sekä myseelikalvojen vetolujuus, venymä ja kimmokerroin määritettiin. Näitä arvoja verrattiin kyseisten sienikantojen geneettisiin eroihin.

Tämä työ tarjosi kattavien analyysimenetelmien kautta katsauksen myseelikalvojen tuotantoon sekä niiden materiaalio minaisuuksiin. Tämä työ edesauttoi soluseinän polysakkaridipitoisuuden määritysmenetelmän kehitystä. Näytteiden ja verrokkien väliset erot myseelin mikrorakenteessa olivat vähäisiä. Villikantojen (Rut-C30 ja QM9414) ja niiden suorien johdannaiskantojen sienirihmat olivat pidempiä ja haarautuiva vähemmän kuin enemmän geneettisesti muokattujen kantojen ja näiden johdannaiskantojen sienirihmat. Tulosten perusteella geneettinen muokkaus vähensi tutkittujen sienikantojen soluseinien polysakkaridipitoisuutta. Fyysisten ja mekaanisten ominaisuuksien tulokset olivat vähemmän yhteneviä, ja niissä olikin viitteitä sekä parannetuista että heikennetyistä vaikutuksista. Tämä työ pohjustaa uudenlaisten myseelimateriaalien kehitytstä. Myseelimateriaaleiden tulevaisuus näyttääkin erittäin valoisalta.

Avainsanat sienirihmasto, myseeli, rihmamaiset sienet, rihmasienet, hyyfi, sienirihma, soluseinän eheyden viestinvälitysketju, Trichoderma reesei, kitiini, glukaani, galaktomannaani, polysakkaridi, soluseinän koostumus, soluseinän rasitus, biosynteesi, biovalmistus, hydrofobiini, resiliini, silkki, kitinaasi, fysikaaliset ja mekaaniset ominaisuudet, tiheys, vetolujuus, venymä, kimmokerroin, proteiinin tuotto, geenin ilmentyminen, RUT-C30, QM9414

Preface

I am thankful for all the support I got along the way of the master's thesis project both from work and home. My advisor doctor Géza Szilvay was very flexible with me and provided support when it was needed, even outside the common working hours. He put a lot of effort in reviewing my thesis, helping me with all the practical things and finding the correct people. My supervisor professor Markus Linder ensured that the thesis would deliver and meet the scientific expectations. My manager doctor Arja Paananen always ensured my well-being at work. My former manager doctor Hannes Orelma and former tutor Vesa Kunnari in a different team at VTT greatly assisted me in getting the master's thesis position.

I would like to thank every party at VTT and Aalto University who helped me with the practical execution of this work. Anniina Valtonen helped me with shake flask cultivations, biomass treatment and microbial analysis, while Pauliina Ahokas helped me with film fabrication and tensile measurements. Tino Koponen and Airi Hyrkäs helped me with cell wall polysaccharide content analysis, while Dinara Bozzhigitova, Riitta Suihkonen and Gabriella Molnar helped me with protein expression analyses, and Timo Kaljunen and Vuokko Liukkonen helped me with tensile measurements.

Of course, this would not have been accomplishable without the support of the lovely Jenny Manninen at home and my amazing family that is by now mostly scattered away from my hometown of Hanko but that is even more essential in my life now than ever. It was especially helpful to receive peer support from a sister of mine Jenni Peuhkurinen, who is currently carrying out her master's thesis at University of Turku.

This thesis is devoted to my beloved mother Hannele Peuhkurinen, who passed away last year, and to my dear brother Juho Karppinen, who passed away two decades ago. My mother, who worked as an accomplished and long-standing laboratory technician, inspired me to pursue a career in chemical industry. My brother was always defending others. And here we are now.

Lauri Peuhkurinen
Espoo, 14.11.2022

Table of contents

Symbols and abbreviations

Symbols

M_{MS}	molar mass of a monosaccharide (g/mol)
M_{MS1}	molar mass of an acetylated monosaccharide (g/mol)
M_{MS2}	molar mass of a deacetylated monosaccharide (g/mol)
M_{MU}	molar mass of a monomer unit (g/mol)
m_{PS}	mass of a polysaccharide (g)
M_W	molar mass of water (g/mol)
n_{MS}	amount of substance of a monosaccharide (mol)
r_1	acetylation ratio (%)
r_2	deacetylation ratio (%)

Abbreviations

BC	bacterial cellulose
CBM	cellulose binding module
CMF	cellulose microfibril(s)
CWI	Cell Wall Integrity
dCBM	double cellulose binding module
DDIW	distilled deionized water
EPS	expanded polystyrene
ER	endoplasmic reticulum
GPI	glycosylphosphatidylinositol
HPIC	high pressure ion chromatograph
IQR	interquartile range
LSB	Laemmli Sample Buffer
MWM	molecular weight marker
P(3HB)	poly(3-hydroxybutyrate)
PU	polyurethane
PVC	poly(vinyl chloride)
RLP	resilin-like protein
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TBS	tris-buffered saline
TBST	tris-buffered saline Tween
TrMM	*Trichoderma* minimal medium
UPR	unfolded protein response
UTS	ultimate tensile strength

1 Introduction

Materials are everywhere, and they are even needed to enable essentially non-material things. Therefore, energy-efficient material solutions, whose whole life cycle have carefully been considered, are needed. Recyclability, degradability, and sustainability go hand in hand in circular economy, and they should be considered as a case-by-case basis since there is no solution that fits all. Sustainability does not only involve the degradability or the source but also the longevity of any given material: bio-based or biodegradable does not necessarily mean more sustainable since the material might perform worse than a fossil-based or non-degradable material in the intended application or at any point in that material's life cycle (Mazhandu et al., 2020; Niaounakis, 2015).

Conventional material solutions have featured materials, such as fossil-based polystyrene as a multipurpose material and concrete as a building material, that generate environmental externalities during their life cycle such as a high carbon footprint (Meyer et al., 2020; Niaounakis, 2015). Many current materials entail sustainability and environmental issues, and materials of the future should provide environmentally conscious solutions. Nature has always been a great inspiration for green circular materials and that is also how biomimetics comes into the picture of the current material development. Biomimetics takes cues from the evolution and the whole life cycle of materials in the nature by observing various ecosystems in the whole range of the biosphere (Kumar et al., 2020; Fratzl, 2007).

There comes a point in the life of all materials when it becomes obsolete, and a need arises to convert it back to energy or to various building block molecules. Fungi are the primary decomposers of waste in the nature due to their capability to form multicellular mycelium networks that can penetrate large pieces of material and while breaking down biomass with the help of high enzyme secretion capabilities (Talbot et al., 2008). Mycelium produced by growing filamentous fungi is a compelling piece of the puzzle of sustainability, especially when it comes to the replacement of certain non-woven fabrics such as animal leather, which have inherent challenges in the material's life cycle, with more holistic material solutions (Wijayarathna et al., 2022; Hildebrandt et al., 2021; Jones et al., 2021; Vandelook et al., 2021; China et al., 2020; Qua, 2019). Mycelium is the vegetative part of filamentous fungi and

mycelium is thus inherently biofabricated and biodegradable (Jones et al., 2021; Webster and Weber, 2007). A multitude of mycelium materials are discussed later in this work.

Despite the advances in genetic engineering and successful demonstrations on how these affect the expression of certain proteins and cellular composition, the relationship between genetics and material properties of mycelium has not yet been addressed thoroughly. Understanding the relationship would enable a more wide and effective use of mycelium as a material, and enhancing, adjusting, and balancing of its material properties for any special needs of a specific intended application. This is the reason why the production of mycelium with various *Trichoderma reesei* strain mutants was studied in this work by comparing the material properties and the polysaccharide content of the produced mycelium films to the genetic differences of the strains. This work laid the groundwork for understanding the relationship between genetics and material properties of mycelium and the experimental limits of used analyses. As a basis for this work, an overview is given both on cell wall composition and regulation in filamentous fungi, and on fungal mycelium materials contrasted with other current commercial or currently researched material solutions in relevant fields.

2 Filamentous fungi and their cell wall

Mycelium is the living network-like cellular structure of filamentous fungi. Establishing the background for fungi and their cell wall is important for understanding interactions and material properties of mycelium. Fungi is a huge taxonomic kingdom with estimated number of species around 1.5 million as of year 2001, ascomycota being the biggest with the estimated number of species between 300 000 and 600 000 (Webster and Weber, 2007). Hence fungi provide a huge potential genetic pool that inspires as it is, and whose benefits could be more efficiently harnessed and expanded with smart genetic engineering, as will be discussed later in this work. This chapter first provides a background for filamentous fungi and then goes into their cell wall components.

Fungi can occur either as unicellular yeasts that divide by binary fission or budding, as multicellular filamentous fungi expanding by ever-branching septate or aseptate hyphae, or as dimorphic hybrids capable of switching between these two growing states (Figure 1A) (Haneef et al., 2017; Webster and Weber, 2007). Unlike yeasts, filamentous fungi have a long fibrous connected structure of hyphae that together build up the mycelium (Webster and Weber, 2007). A hypha is formed by one or more fungal cells, and it is surrounded by a tubular cell wall growing only at its tip in a specific angle to the growth axis (Webster and Weber, 2007; Moore and Novak, 2002). A fruiting body is the multicellular part of fungi capable of sexual reproduction with spores, while vegetative mycelium growth and asexual spore production can happen without a fruiting body (Webster and Weber, 2007). Mycelium is in essence the non-fruiting body and the non-reproducing vegetative part of filamentous fungi, although structurally the line between these two is commonly very ambiguous, as can be seen in Figure 1B.

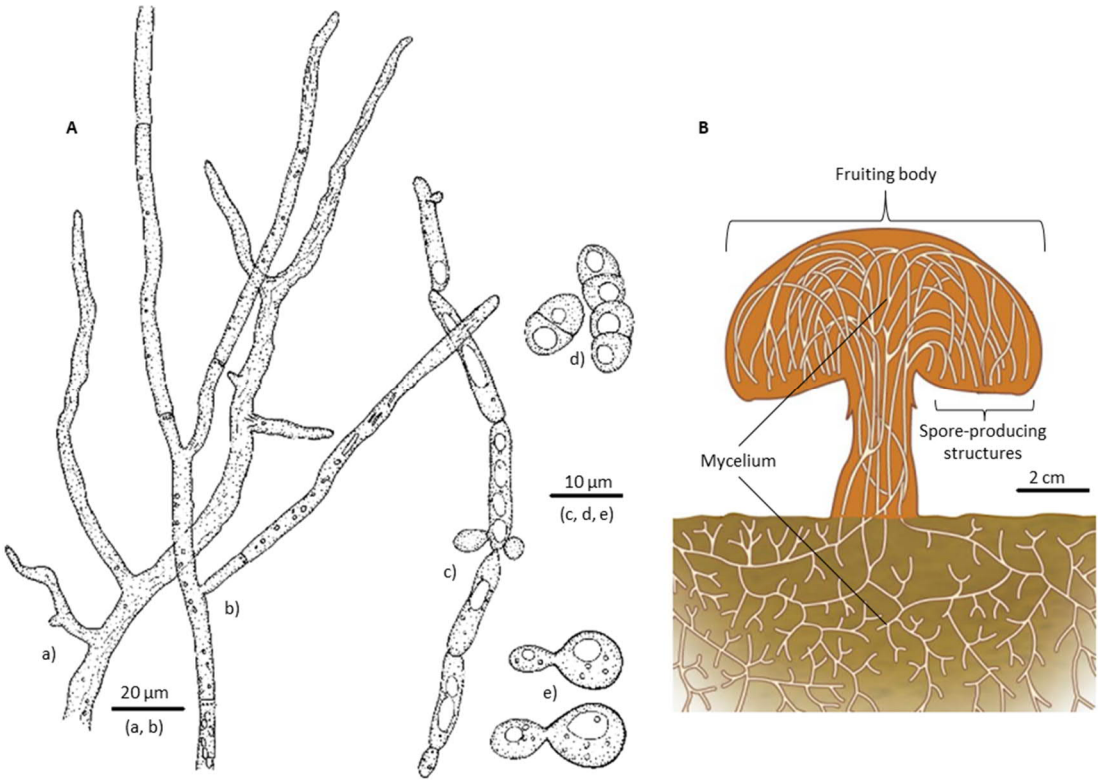


Figure 1. Growth forms of fungi (A) and macrostructures in fungi (B). Filamentous fungi grow as mycelium formed by either aseptate hyphae (a) or septate hyphae (b), while yeast cells divide by binary fission (d) or budding (e). Some fungi can form pseudohyphae (c) that are morphologically between yeast cells and true hyphae. Mycelium (f) is the vegetative part of fungi and while fruiting body (g) bears sexual spore-producing structures (h). Not all fungi form fruiting bodies. A) is adapted from Webster and Weber (2007) and B) is adapted from Rathore et al. (2019).

This thesis focused on the filamentous fungi of the largest fungal division Ascomycota and more specifically on the species *T. reesei*, which was also used in the experimental work. Fungi of the always filamentously growing *Trichoderma* genus are significant players in parasitism of plants and humans among other animals and thus the structure and the regulation of their cell wall has been an important research topic at least since the 90s (Atanasova et al., 2018; Wang et al., 2018; Gow et al., 2017; Rogg et al., 2011; Aufauvre-Brown et al., 1997). *T. reesei* has had a long history in industrial use since it was first discovered in the mid-20th century as the extensive cellulase producing wildtype QM6a strain that was further developed into strains, like Rut-C30 and QM9414, with improved protein production capabilities (Peterson and Nevalainen, 2012).

In the higher fungi (Ascomycota and Basidiomycota), hyphae can assemble into fruiting structures producing and bearing the sexually produced spores. The inner vegetative portion of these fruiting bodies is referred as trama (sometimes referred as the context tissue) and

it has similar structure to mycelium. The structural properties of trama can vary from species to species, just like for mycelium, and from region to region. Spores can also be produced asexually or without fruiting bodies, as is the case with yeast *Saccharomyces cerevisiae*. Only a very few fungi get by without spores thus only relying on mycelium (Webster and Weber, 2007). Fungal cell wall is the main structural constituent of mycelium (Gow et al., 2017; Webster and Weber, 2007). The cell wall is the outer-most line of defence for the fungi sustaining the morphology of the cell and intermediating external stimuli into the cell (Yoshimi et al., 2022). The cell wall was considered inert for a long time although it has very much proven to be a highly dynamic organelle (Latgé, 2007).

2.1 Cell wall components

Cell wall composition of filamentous fungi likely affects the material properties of mycelium. The cell wall of fungi plays a significant role in the interactions between the fungal cell and its environment, and it resists the high turgor pressure of the cells and retains the form. Fungal cell wall must adjust to the environmental changes to survive. This adaptability is enabled via a highly complex regulation network that controls various aspects of produced cellular components (Kappel and Gruber, 2020; Free, 2013). The cell wall constitutes approximately a third of the whole dry weight of filamentous fungi (Kappel and Gruber, 2020). The cell wall components of filamentous fungi include various polysaccharides, cell wall associated proteins and lipids (Kappel and Gruber, 2020; Free, 2013; Feofilova, 2010), as can be seen in Figure 2.

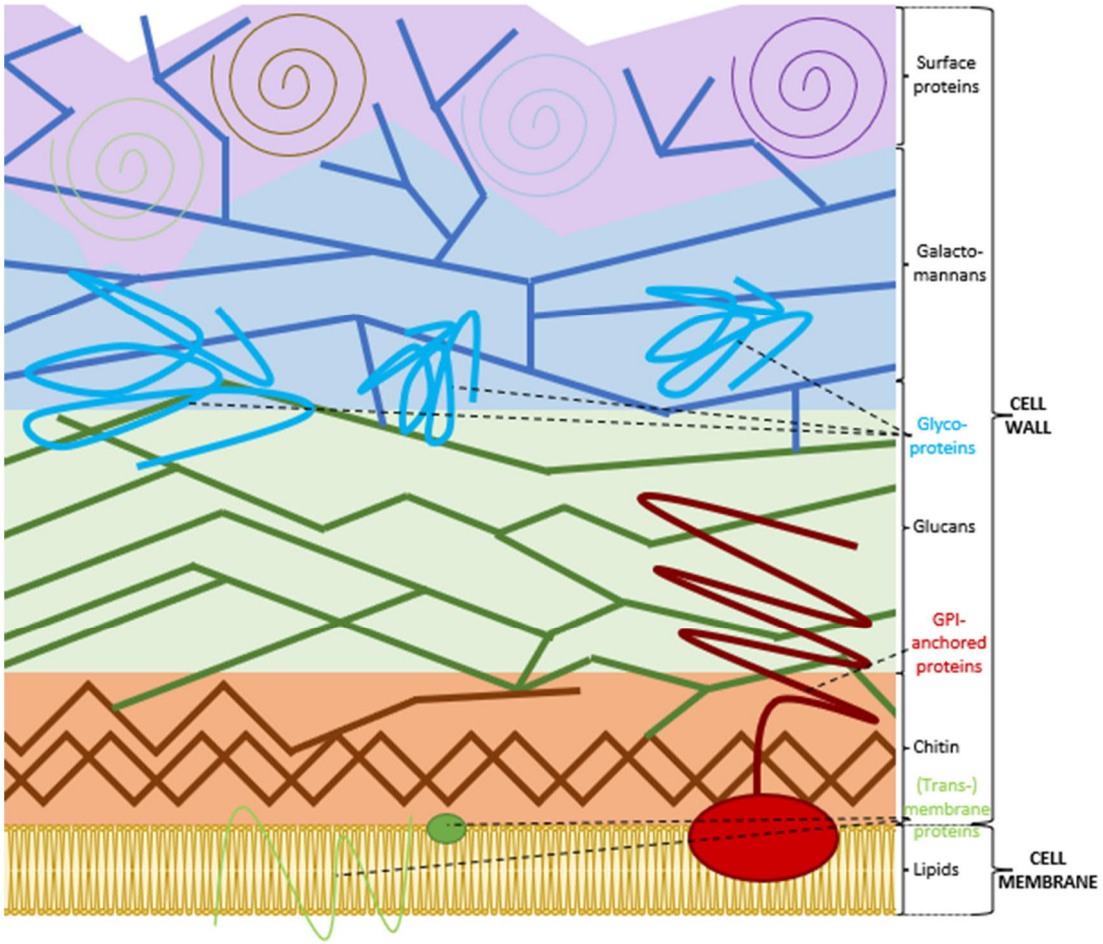


Figure 2. Cell wall structure of *Trichoderma reesei* (based on Kappel and Gruber (2020)).

2.1.1 Polysaccharides

Glucans, chitin, and galactomannans are the most notable polysaccharides in the cell wall of filamentous fungi and in general these are the major components in the cell wall in addition to varying amounts of proteins (Kappel and Gruber, 2020; Free, 2013). Polysaccharides are a wide group of natural carbohydrate compounds, sometimes referred as glycans (Avenas, 2013; Brock et al., 2003). The core structural scaffold of fungal cell walls is provided by β-1,3-glucan and chitin microfibrils (Kappel and Gruber, 2020).

Fungal cell walls contain various alpha and beta glucans. Glucan is a polysaccharide composed of glucose monomers (Ruiz-Herrera and Ortiz-Castellanos, 2019). β-1,3-glucan is the most prevalent glucan in fungal cell wall linked with varying amounts of β-1,6-glucan branches and in rare cases β-1,4-glucan branches (Kappel and Gruber, 2020; Ruiz-Herrera and Ortiz-Castellanos, 2019). Glycogen, formed by linearly α-(1-4)-linked and branching α-(1-

6)-linked glucose subunits, is the most abundant alpha glucan in fungi, but it is merely an intracellular energy reservoir and is not directly related to mechanical properties of the fungal cells. While there are structural alpha glucans in fungal cell walls, their role is minor (Ruiz-Herrera and Ortiz-Castellanos, 2019). β-1-3-glucan microfibrils provide general structure, and cross-linking β-1,3-glucan and β-1,6-glucan network provides support to the microfibrils (Free, 2013). Glucans are synthesised by various alpha and beta glucan synthases on the plasma membrane. An extensive review of fungal cell wall glucans has been compiled by Ruiz-Herrera and Ortiz-Castellanos (2019), although the cell wall of *Trichoderma* is poorly understood.

Chitin has three naturally occurring allomorphs and in fungi, only the most substantial and the antiparallelly chained form, α-chitin, is found. Chitin is a polysaccharide where glucose-derived N-acetylglucosamine or glucosamine monomers are joined via β-(1-4) linkages to form the polymer (Kappel and Gruber, 2020). This glycan molecule is called chitosan if most of the monomer units are glucosamine (Kappel and Gruber, 2020). Chitin has high tensile strength and great flexibility due to its intrachain hydrogen bonds (Kappel and Gruber, 2020). Chitin and chitosan provide integrity and rigidity to the cell wall and protection against various environmental stress factors (Kappel and Gruber, 2020; Feofilova, 2010). Chitin is synthesised on the cell membrane through the action of chitin synthase enzymes (Kappel and Gruber, 2020).

Galactomannans, which consist of mannose backbone with branching galactose side chains, are specific to cell walls of filamentous fungi and a significant component of the outer layer of the cell wall of *T. reesei* (Kappel and Gruber, 2020). Neither the function nor the biosynthesis of galactomannans is well understood (Fontaine and Latgé, 2020). The addition of mannan and galactomannan to form glycoproteins and their biosynthesis happen in the endoplasmic reticulum (ER) and Golgi apparatus by mannosyl- and galactofuranosyltransferases (Fontaine and Latgé, 2020; Gow et al., 2017; Free, 2013).

Dry cell wall of filamentous fungi *Trichoderma spp.* contains between 65 and 90 % of β-1,3-glucan and between 0.5 and 5.0 % of chitin. Chitosan is only present in small ratios and the amount has typically been included in chitin (Kappel and Gruber, 2020; Free, 2013). The cell wall of another filamentous fungus *Aspergillus fumigatus* has been reported to contain

between 20 and 25 % of galactomannans (Free, 2013; Gastebois et al., 2009), but the amount of galactomannan in *Trichoderma* spp. is not well known.

2.1.2 Proteins

Kappel and Gruber, (2020) reported that around 30 % of the total cell wall dry weight of *T. reesei* is made of proteins. Proteins, biomolecules consisting of one or more amino acids linked by peptide bonds, are very integral compounds for all living organisms (Brock et al., 2003). Cell wall associated proteins include proteins that are anchored to the cell wall or secreted to the extracellular matrix (Free, 2013). Some proteins are covalently linked to glycan molecules in post-translational modification steps and are thus classified as glycoproteins (Gamblin et al., 2009). There are diverse transmembrane and membrane proteins – including enzymes, receptor proteins and transportation proteins – embedded in the fungal cell membrane interfacing with the cell wall, many of which have an important role in cell regulation, transportation, or specific enzyme activity (Brock et al., 2003; Moore and Novak, 2002). Proteins that are linked to the glucan-chitin network of the cell wall of filamentous fungi via glycosylphosphatidylinositol (GPI) anchors are called GPI-anchored proteins (Kappel and Gruber, 2020; Free, 2013).

Various cell wall surface proteins including flocculins, agglutinins, hydrophobins and cerato-platanins are bound to the surface of the cell wall (Kappel and Gruber, 2020). *T. reesei* has a high number of hydrophobins present in the outer layer of the cell wall (Kappel and Gruber, 2020). Hydrophobins can cover glucans of aerial hyphae and conidia by transforming a hydrophilic surface to a hydrophobic surface (Kappel and Gruber, 2020). Cerato-platanins are a form of hydrophobin-like proteins that play a role in the interaction with host in plant pathogenic fungi (Bacceli, 2015). The extensively studied hydrophobins HFBI and HFBII are the *T. reesei* native proteins investigated in this work in addition to various recombinant proteins, and those are discussed further in Chapter 5.1. Melanins consisting of phenolic or indolic compounds are hydrophobic high-molecular-weight pigments that, in fungal cells, form protective layers against various environmental hazards (Kappel and Gruber, 2020; Gow et al., 2017). In *T. reesei*, a polyketide synthase gene *pks4* regulates the pigmentation of conidia, and the absence of the gene causes instability of the conidial wall and reduces the antagonistic capabilities against other fungi (Kappel and Gruber, 2020).

2.1.3 Lipids

Lipids were for a long time seen as impurities in the cell wall – just like proteins at some point –(Ruiz-Herrera, 2016) possibly explaining why lipids are still the least studied relevant macromolecule group in fungal cell walls (Ruiz-Herrera, 2016). Lipids are hydrophobic or amphiphilic macromolecules soluble in water. In fungi, lipids are mainly constructed from fatty acids, which in turn are hydrophilic carboxylic acids combined with a saturated or non-saturated hydrophobic aliphatic chain (Brock et al., 2003). While the cell membrane directly below the fungal cell wall is formed by a lipid bilayer (Brock et al., 2003), the contribution of lipids (more specifically the lipids outside the lipid bilayer of the cell membrane) to the total fungal cell wall dry weight is roughly between 1 and 10 % depending on the fungal species in question (Ruiz-Herrera, 2016). Functions of lipids in fungal cell wall are not well understood but it is suspected that those are related to their hydrophobic properties and to providing protection against dehydration (Ruiz-Herrera, 2016).

2.2 Cell wall regulation

The cell wall components and their properties are controlled by genes that are either species-specific or conserved across all fungi. Hundreds of genes are encoding a highly complex system where the cell wall composition, assembly and integrity are tightly controlled enabling a highly adaptive cell wall formation. This mechanism of cellular responses and signalling that allows the fungus to re-balance and adapt its cell wall to changes in various environmental conditions, stress, and stimuli, is called the Cell Wall Integrity (CWI) pathway. The first part of the CWI pathway is various cell surface sensor proteins integrated in the cell wall detecting changes in the cell wall structure, the second part is the transduction of that information signal inside the cell by signal proteins, and the third part is the transcription factors regulating the expression of the genes involved in CWI pathway based on the received signal molecules (Yoshimi et al., 2022; Malavazi et al., 2014; Fuchs and Mylonakis, 2009).

The CWI pathway has been most extensively described for the baker's yeast (*Saccharomyces cerevisiae*) (Malavazi et al., 2014; Levin, 2011) as a model fungal organism, while filamentous fungi has received less attention. It has been noted that many parts of the CWI pathway are conserved between filamentous fungi and yeast (Yoshimi et al., 2022; Malavazi et al., 2014; Fuchs and Mylonakis, 2009). The biggest differences between the CWI pathway of *S.*

cerevisiae and filamentous fungi are caused by the complete lack of α-1,3 glucan (and related synthases and regulation systems) and hyphae (and a polarized growth) in *S. cerevisiae* (Malavazi et al., 2014).

The CWI pathway of filamentous fungi is highly interconnected to other regulation mechanisms and signal pathways, such as the unfolded protein response (UPR), the general amino acid control and the ER associated degradation of proteins, and mitogen-activated protein kinase cascades. Malavazi et al. (2014) investigated the cross talk between the CWI pathway and the UPR pathway focusing on *T. reesei* and *Aspergilli*. They noted that the UPR is extremely conserved in filamentous fungi, and that the UPR compensates for the compromises in the CWI pathway. Furthermore, the UPR assists in the coordination of cell wall related genes and affects the secretory and cell wall homeostasis in filamentous fungi. Both the UPR and the CWI are regulated by ER stressors, such as dithiothreitol and tunicamycin, and cell wall stressors, such as cell wall destabilizing agents congo red and calcofluor white (Malavazi et al., 2014). A common and an expected response to perturbation in the cell wall is an increase in the genetic response to produce enzymes responsible for the cell wall structures, such as chitin and beta glucan synthases in *T. viride* (Kappel et al., 2020).

The pathways and mechanisms related to fungal cell walls are diverse and multifaceted. Thus, they provide an interesting albeit a challenging target for genetic engineering. It has been identified that the CWI signalling could be utilized in controlling the fungal growth, such as the formation of hyphal pellets for a more stable industrial fungal fermentation (Yoshimi et al., 2022). Thus, It is likely that the knowledge on the cell wall regulation pathways and genetic engineering of the mechanisms in those pathways could be utilized in biotechnology and fungal materials, as well.

3 Fungal mycelium materials

There is a growing need to replace conventional petrochemical-based or animal-based materials with renewable and animal-free material solutions to reduce the environmental impact during the whole life cycles of materials. Bio-based materials made from renewable raw materials such as fungal materials provide a potential answer to this need. This chapter first discusses the history of fungal materials and past and current research and commercial applications on various types of fungal materials before proceeding to genetic engineering of fungi with potentially enhanced material properties for mycelium.

Fungi have been used as material for a long time and there is direct evidence for a common history between fungi and humans for at least 5000 years: the mummified "Ötzi the ice man", who was found in the Alps in 1991 and estimated to have deceased in 3230 BC, possessed pieces of *Piptoporus betulinus* and *Fomes fomentarius* fruiting body tinder materials, also known as amadou or tapló, for fire making (Gandia et al., 2021; Peintner et al., 1998). Gandia et al. (2021) composed that indigenous people of Europe and North America had commonly used *Fomitopsis betulina* fruiting bodies in medicinal purposes. In a more advanced material role, *F. fomentarius* fruiting bodies were traditionally utilized in Europe in crafting felt-like fungal fabric known as German felt, which could be used in absorbent wound dressings and clothing garments. Another age-old fungal material is a fabric mat produced out of *Laricifomes officinalis* fruiting body utilized by indigenous people of North America (Blanchette et al., 2021).

Although fungi have been used as a material for millennia, there has been only little research and development activity regarding fungal materials over the years. However, the number of patents and publications for fungal materials – especially for fungal fabrics – has substantially increased in the last decade alone (Jones et al., 2021; Cerimi et al., 2019). The research and development process for fungal materials has recently been critically analysed with various focus points: Meyer (2022) highlighted the importance of the interdisciplinary and transdisciplinary collaboration of designers, engineers, and biologists in the fabrication of fungal materials, while van den Brandhof and Wösten (2022) stressed out the importance of risk assessment for the whole life cycle of fungal materials: the fungal species used for the

material production, its pathogenicity, and all hazards related to the raw material extraction, the material manufacture, the product manufacture, the use and the end-of-life.

Most of the research and development efforts have focused on cultured mycelium, while whole fruiting bodies and trama tissue have commonly only been utilized in more traditional fungal material applications or more fundamental level research on mycelium, since the production has not been as easy to be automated for fruiting bodies (Gandia et al., 2021; Jones et al., 2021; Vandelook et al., 2021; Webster and Weber, 2007). Mycelium can be cultured by several fermentation methods depending on the intended material type and application, the most common being solid-state fermentation where the mycelium grows in a flatbed of moist substrate colonizing it (Vandelook et al., 2021; M. P. Jones et al., 2019). One key advantage of fruiting bodies as a mycelium source over cultured mycelium is the sectional differences in material properties within trama tissue widening the possible applications: Müller et al. (2021) described material properties of a fruiting body of a well-known bracket fungi *F. fomentarius* noting that it had properties of anisotropic lightweight foams. Regardless of the production and acquisition method for mycelium, various agricultural and forestry by-products can be upcycled into mycelium thereby decreasing the amount of possible waste streams and even possibly enhancing the propertis of the material (Sisti et al., 2021; Joshi et al., 2020; M. Jones et al., 2018, 2019; M. P. Jones et al., 2018).

Most fungal materials can essentially be classified as mycelium materials regardless of the source or the acquisition method. In this thesis, fungal materials were divided into mycelium composites, mycelium foams, and mycelium non-woven fabrics, although there is certainly cross-over between these categories. A multitude of commercial applications for mycelium materials have already entered the market while many more are currently in development worldwide (Jones et al., 2021; Vandelook et al., 2021), as shall be discussed next.

3.1 Composites

Mycelium, as a material component, is a polymeric composite of various structural biopolymers (Haneef et al., 2017). Composites are a diverse group of materials that can be defined in various ways depending on the applications (Fakirov, 2015; Shackelford, 2015). A composite consists of two or more components that have significantly different chemical or

physical structure which remain separate and distinguishable in the final structure as matrix and reinforcement components (Shackelford, 2015). Thus, additives alone (like sorbitol or glycerol) do not turn a material into a composite. In a composite, mycelium typically serves as the matrix binding to a lignocellulosic waste substrate (Vandelook et al., 2021). Mycelium composite materials have been widely studied, especially form the viewpoint of packaging and construction materials, and thus keen readers are advised to check the review articles from Jones et al. (2017) and Attias et al. (2020).

The cultivation of mycelium is a great way of upcycling of side and waste streams from forest, agriculture, and food industries. Most typically solid-state fermentation is used to grow mycelium around solid forestry or agricultural by-product particles – such as sawdust, wheat straws or blackstrap molasses – that act as both nutritious substrate for the fungus and as the reinforcement component of the composite material. After the mycelium has fully colonized the substrate, the process is stopped by heat inactivation. The used substrate particles can influence the material properties through their material characteristics but also through influencing the growth of the fungus (Jones et al., 2021). Sisti et al. (2021) reported that wheat bran was an excellent growth medium for mycelium composite promoting a fast growth, composite surface hydrophobicity and homogeneity of the composite. A more unusual example of a side stream upcycling into a less common product was conducted by Silverman et al. (2020), when they manufactured a shoe sole composite out of mycelium integrated within chicken feather waste, although especially the flexibility of the composite material needs to be enhanced to reach their future goal of a shoe made entirely out of mycelium. Rafiee et al. (2021) conducted a comprehensive study on how bioreactor design affects the mycelium growth and the ability to control mycelium microenvironment for the development of an optimal and sustainable automated process to address the higher labour cost involved with more manual mycelium composite manufacturing when compared to current fossil-based polymer alternatives.

In addition to the upcycling of side streams, the use of mycelium in a composite can bring various benefits, such as enhanced fire safety, thermal, impact and sound insulation properties and biodegradability. Jones et al. (2018) studied the fire-retardant properties of mycelium-biomass composites and found that a wheat grain – mycelium composite demonstrated more charring and thus it provided more thermal insulation than wheat grain

composites with polymers such as poly(methyl methacrylate) and polylactic acid as the matrix. Sivaprasad et al. (2021) reported that although the water absorption capacity for a *Pleurotus ostreatus* fungal mycelium – saw dust – coir pith composite was higher than for expanded polystyrene (EPS) and not ideal for packaging, the superior compression energy absorption and flexural tensile capabilities for the mycelium in comparison to EPS in addition biodegradability, sufficient shelf-life of at least two months, sound and thermal insulation properties and self-extinguishing fire-retardant properties, made the mycelium composite an optimal candidate for packaging of dry goods.

In commercial use, mycelium composite materials are typically found either in construction or packaging applications. Good examples are the acoustic panels and the floor tiles produced by Italian mogu (2022c, 2022b, 2022a) and a mycelium packaging material and a mycelium hemp blend construction material both produced with the MycoComposite material by the American Ecovative Design LLC (2022a).

3.2 Foam materials

Mycelium foams consist of long entangled hyphae and depending on the manufacturing process they are either classifiable as a composite or a pure material. Foam materials are a two-phase cellular structure of gas pockets enclosed within a liquid or solid layers of thin film (Weaire and Hutzler, 1999). Mycelium foams are manufactured by enabling the formation of aerial hyphae while limiting the spore formation by carefully controlling the carbon dioxide levels, humidity and temperature. Spores and fruiting bodies could cause undesired inconsistencies in fungal material structure and properties (Jones et al., 2021; Vandelook et al., 2021).

Mycelium foams are used in packaging and in construction mainly in a composite form, and the current commercial applications for pure mycelium foam materials are limited (Jones et al., 2021; Vandelook et al., 2021). One example of a mycelium foam material in commercial use is Forager foam produced by Ecovative Design LLC (2022b). Curiously, pure mycelium foams are a common raw material source for mycelium non-woven fabrics (Jones et al., 2021; Vandelook et al., 2021). The before mentioned Forager foam is produced by so called AirMycelium technology that can be used for mycelium fabric production such as the Forager

hide (Ecovative Design LLC, 2022b). Another mycelium foam production technology is by American Bolt Threads (2022), which has been used in the production of their leather-like mycelium material Mylo that has commercially been used in shoes, yoga mats and handbags (Bolt Threads, 2022).

3.3 Non-woven fabrics and bioleather

Mycelium as a non-woven fabric usually involves leather-like material fabrication (Jones et al., 2021). Non-woven fabrics are flat porous sheets consisting of a network of fibres held together either by the attractive forces between the fibres or by a chemical adhesive via mechanical thermal or chemical treatments, but not by weaving or knitting from yarn made out of fibres (Syed et al., 2015). Leather is a widely used and a very durable material that is bio-based but has hindered degradability after tanning (China et al., 2020). Leather-like materials are either bio-based, sometimes referred as bioleather (Bustillos et al., 2020; García and Prieto, 2019), or synthetic materials that have leather-like physical and mechanical properties (Jones et al., 2021). The most dominant and high-performing current leather-like alternatives for animal leather are composed of petroleum-based polyurethane (PU) or poly(vinyl chloride) (PVC) polymers, but an increasing number of companies are utilizing mycelium in their material applications (Jones et al., 2021). Mycelium was studied as a leather-like material in the experiments of this thesis, as well.

Animal leather materials have been compared to synthetic and other non-animal leather-like materials to get a better grasp on the requirements for leather-like materials. Total Higg Materials Sustainability Index and thus the environmental impact of bovine leather is over three times higher than for synthetic PU or PVC leather-like alternatives, if leather is considered as a co-product of meat and milk and not a by-product (Jones et al., 2021). However, it would not be sustainable to simply replace animal leather immediately without a broader examination. Almost all of the global animal leather is obtained from animals raised primarily for milk or meat production (Bustillos et al., 2020; Pelletier et al., 2019), while at the same time the global meat consumption is still increasing (González et al., 2020). Although sales of vegan products have been steadily increasing (Boukid et al., 2022), it is unlikely that it will have an impact on the animal hide availability on the global scale in the near future. Additionally, as Hildebrandt et al. (2021) pointed out in their study, careful

optimisation in manufacturing processes is needed so that ecological advantages of bio-based leather-like fabrics are not negated. Mycelium has potential economically, but it is not achieved effortlessly.

Furthermore, the economic aspects are promising for fungal bioleather. Jones et al. (2021) calculated that the production of raw materials for leather-like materials (with fixed material dimensions and a certain minimal production capacity) could be at least 24 times cheaper in form of mycelium than in form of PU or PVC polymers, and at least 29 times cheaper than in form of unprocessed bovine hides. Not to mention that the tanning process of animal hide is very chemically and energetically intensive process in comparison to processing of synthetic and especially fungal leather-like materials even with chromium-free tanning methods with vegetable tannins and aluminium sulfate (Raman et al., 2022; China et al., 2020). Mycelium is economically very feasibly to produce with capable production technology.

There are multiple compelling and detailed examples on cultured mycelium and their material properties. In Sweden, scientists have been developing a fungal leather-like material from bread waste in effort to tackle the issues of food and clothing waste simultaneously. They cultivated filamentous fungus *Rhizopus delemar* in submerged fermentation and used a wet-laid technique to obtain mycelium sheets that were then treated with glycerol, biobased binders and ultimately with vegetable tannin of chestnut wood. (Wijayarathna et al., 2022). Another Swedish group wet-spun grinded and alkali hydrolysed mycelium hydrogel – obtained from the same species and cultivation method that Wijayarathna et al. (2022) utilized – into monofilaments. They did not identify any cytotoxicity against human dermal fibroblasts and thus the material could be used as a biomedical textile. The wet-spun filaments had Young's modulus of 4.97 GPa and Tensile strength of up to 69.5 MPa (Svensson et al., 2021, 2022). Haneef et al. (2017) observed that *Ganoderma lucidum* and *Pleurotus ostreatus* mycelium films were less stiff, less brittle, and more hydrophobic than bacterial cellulose (BC) and poly(3-hydroxybutyrate) (P3HB), which are two other typical self-growing microbially produced materials. Furthermore, they reported that the nutrient source affected the structural properties of the mycelium films, while P3HB and BC were mainly affected in their yields and molecular weights. Bustillos et al. (2020) studied mechanical, thermal, and chemical characteristics of leather-like *Phellinus ellipsoideus* mycelium and

noted that it was thermally stable at up to 250 °C and it had a tensile strength of 1.2 MPa and strain at break of 101 %, making it a very flexible material.

Commercial product examples of bioleather materials were collected in Table 1. Many materials were obtained from waste or side streams of fruits and vegetables, and commonly this saccharose-rich substance was combined with polyurethane, polyester, or a similar polymer, most likely to increase the tensile properties of the material (ADRIANO DI MARTI, 2022b, 2022a; Ananas Anam Ltd., 2022; Fruitleather Rotterdam, 2022; MoEa, 2022; Vegatex Biotech Ltd., 2022; Vegea, 2022). Many companies producing vegetable bioleather materials were based in Italy. There were also collagen-based (Modern Meadow, 2022), and bacterially produced bioleather materials (Malai Eco, 2022). Multiple commercially available fungal bioleather material solutions have emerged from multiple companies (Bolt Threads, 2022; Ecovative Design LLC, 2022b; Grado Zero Espace Research Lab., 2022; Mycotech Lab, 2022; MycoWorks, 2022). A great benefit of fungal bioleather materials is the controllability of the whole manufacturing process from start to beginning and the independency from small-scale only side or waste streams.

Table 1. Commercial applications for mycelium and other non-animal bio-based fabrics.

Product type Product name	Source	Producer	Country	Reference
Bioleather				
Mylo™	Fungi	Bolt Threads	USA, California	a
Forager Hide	Fungi	Ecovative Design LLC	USA, New York	b
Muskin	Fungi	Grado Zero Espace Research Lab.	Italy	c
Mylea	Fungi	Mycotech Lab	Indonesia	d
Reishi™	Fungi	MycoWorks	USA, California	e
Malai	Bacteria	Malai Eco	India	f
AppleSkin™	Apple	Frumat	Italy	g
Vegatex™	Apple	Vegatex Biotech Ltd.	China	h
Desserto®	Cactus	ADRIANO DI MARTI	Mexico	i
Deserttex®	Cactus	ADRIANO DI MARTI	Mexico	j
UltraWer	Corn	Fiscatech	Italy	k
Vegea®	Grape skin	Vegea	Italy	l
Fruitleather Mango Sheet	Mango fruit	Fruitleather Rotterdam	Neatherlands	m
Piñatex®	Pineapple leaves	Ananas Anam Ltd.	UK	n
Zoa™	Collagen	Modern Meadow	USA, New York	o
Bio-Tex™	Collagen	Modern Meadow	USA, New York	o
Biomedical skin				
Bio-Coll@gen™	Collagen	Modern Meadow	USA, New York	o
Textiles				
S.Café®	Coffee with yarn	SINGTEX Industrial CO., Ltd.	Taiwan	p

References: a) Bolt Threads (2022) b) Ecovative Design LLC (2022b) c) Grado Zero Espace Research Lab. (2022) d) Mycotech Lab (2022) e) MycoWorks (2022) f) Malai Eco (2022) g) MoEa (2022) h) Vegatex Biotech Ltd. (2022) i) ADRIANO DI MARTI (2022a) j) ADRIANO DI MARTI (2022b) k) Fiscatech (2022) l) Vegea (2022) m) Fruitleather Rotterdam (2022) n) Ananas Anam Ltd. (2022) o) Modern Meadow (2022) p) SINGTEX Industrial CO. (2022).

Common fungal non-woven material applications involve leather-like fabrics and direct use of mycelium, but additionally the use of mycelium as a polysaccharide-rich source to make paper-like materials has been investigated in recent years. Nawawi et al. (2020) extracted chitin from fruiting bodies of a tree bracket fungi *Daedaleopsis confragosa* and a common mushroom *Agaricus bisporus* and hot-pressed those into fungal chitin nanopapers with superior tensile strength to a crustacean-derived chitin nanopaper. Further benefits of fungal chitin over crustacean-derived chitin are that fungal chitin is free of tropomyosin allergen, readily and steadily available without demineralisation, while the disadvantages include in the lower and impure chitin content and the lack of industrial methods for the extraction and nanopaper manufacturing of fungal chitin (Fazli Wan Nawawi et al., 2019).

3.4 Biotechnology

The production of both native and heterologous proteins via eukaryotic fungi has its benefits over prokaryotic bacteria, since the post-translational modifications of proteins in fungi, such

as disulphide bond formation and glycosylation, more closely resembles those in humans than in bacteria (Lodish et al., 2004). However, despite the similarities, fungi naturally lack some post-translational modification capabilities and have different kind of post-translational modification machinery and routes in comparison to animals. That is why there have been efforts to modify that machinery of certain fungi (like *Pichia pastoris* and *T. reesei*) to meet the specific post-translational modification needs of particular heterologous proteins in medicines for humans (Sunagawa and Igarashi, 2021; Wei et al., 2021; Ward, 2012).

Mycelium has conventionally been seen as a major by-product of industrial fungal bioproduction of enzymes, chemicals, drugs and food components, and the processes have been optimized for the best yields for the desired compounds with as little by-products as possible (Candida et al., 2021; Jones et al., 2021; Vandelook et al., 2021). A production of mycelium material requires a reversed perspective into the fungal bioproduction output. A comprehensive understanding and utilisation of fungal biotechnology could potentially enable sustainable circular economy in development of versatile components for a great variety of materials, as was discussed by Meyer et al. (2020).

Genetic modifications are an important tool in fungal biotechnology to meet the diverse needs of bio-applications, by a creation of new optimized fungal strains. There are several techniques that can be used for fungal genetic modifications, including CRISPR/Cas9 genome editing, homologous recombination and restriction enzyme-based cloning, and transformation methods including protoplast-mediated transformation, electroporation, *agrobacterium*-mediated transformation, biolistic transformation, and shock-wave-mediated transformation (Ullah et al., 2020; Li et al., 2017). Traditional genetic engineering techniques that do not involve direct incorporation of exogenous genetic material into the target genome via transformation, have utilized chemical or UV mutagenesis methods followed by selective steps and, in some cases, by non-selective monitoring steps (Moore and Novak, 2002). Both traditional and modern genetic modification methods have been utilized in the past for building the fungal strains that were used in the experiments of this work.

Material properties of mycelium are influenced by fungal genome, cultivation conditions, additives and co-components and post-processing methods. Bae et al. (2021) demonstrated the effects of cultivation media and carbon sources on the growth characteristics and the physical properties of mycelium originating from various mushrooms species of Polyporales order: Best results in their experiment were obtained with *Ganoderma lucidum* mycelium on either a potato dextrose liquid media or saw-dust based solid media with glycerol and skim milk as nutrient sources illustrating thick hyphae with a diameter of 13 µm. Bustillos et al. (2020) reported that the pH value of the cultivation media affected the dissolution of either protein or polysaccharide components within mycelium to allow adjustable mechanical properties. Mycelium could be coated with plastics such as polylactic acid to increase the durability against abrasion, as was studied by Jones et al. (2021). Appels et al. (2020) reported that the treatment of mycelium with glycerol increased the flexibility (lowered Young's modulus) and the density of the film material eventually causing it to be classified as an elastomer or polymer material instead of being classified as a natural material as the non-treated mycelium films. These studies demonstrated the importance of selecting a correct fungal species and determining optimal production and post-processing methods to manufacture a material with desired properties most efficiently.

It is not well known how genes or production capabilities of various proteins affect the material properties of mycelium. There has been at least one study on the role of a protein production phenotype in cell wall integrity: Zhang et al. (2021) investigated the role of the typical yellow pigment in *T. reesei* in cell wall integrity stability by mutating a uridine auxotrophic strain QM9414 Δ*pyr4* in relation to *sor1* and *sor2* genes (encoding for two different polyketide synthases relevant for the yellow pigment protein) and genes *ypr1* and *ypr2* (encoding for yellow pigment synthase related transcription regulators YPR1 and YPR2, respectively). They reported no negative effects for the studied strains not producing yellow pigment, while the cell wall integrity was more stable and the stress tolerance higher for the yellow pigment deficient strains than for the reference strain and the yellow pigment hyperproducing strains. However, this study did not investigate any material properties of the produced mycelium.

To the author's knowledge, the relationship between singular genetic modifications and mycelium properties has been investigated only once before: Appels et al. (2018) reported

that the hydrophobin gene *sc3* of *Schizophyllum commune* – controlling the production of SC3 hydrophobin – affected the density of mycelium. This find motivated the work of this thesis and was one of the reasons why multiple strains with different hydrophobin production characteristics were studied. Additionally, the existing *T. reesei* strain library was studied instead of developing new strains, to identify possible marginal terms more efficiently for mycelium production. This allowed an enhanced development of a research platform to comprehensively study diverse material properties of mycelium in the future.

4 Aims of the study

In this thesis, the goal was to determine whether certain genes in genetically engineered *T. reesei* strains could affect the mycelial material properties. These strains had been developed earlier and no new strains were generated. This thesis aimed to determine whether single gene changes would affect the mycelium composition and whether these changes would be measurable in the material properties of mycelium.

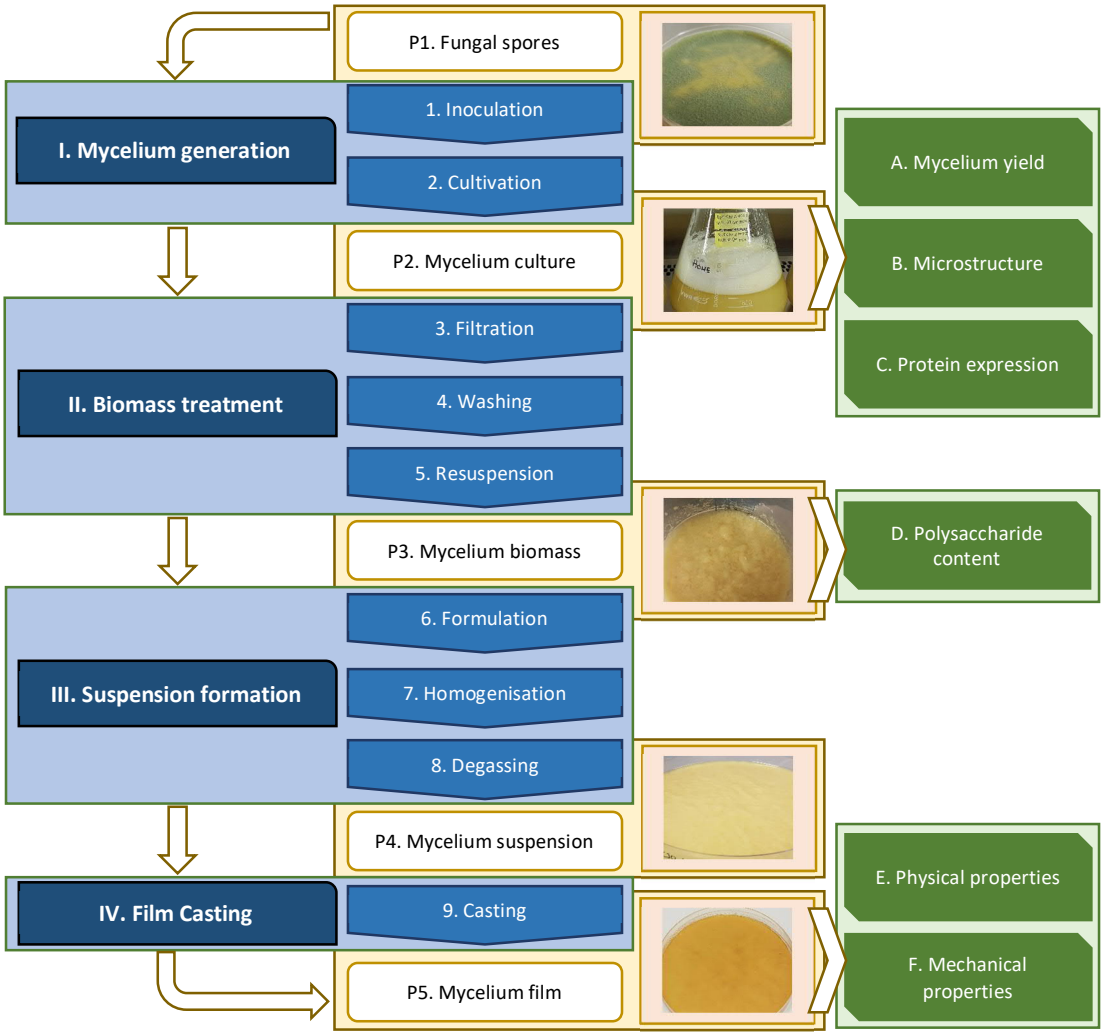
T. reesei strains to be studied were selected and a consistent process route was established for mycelium cultivation, harvesting and film fabrication. Test batches were made with various iterations based on previously patented work (Ahokas et al., 2020) to eliminate excess variables and finally arrive on a suitable process. This work also displayed measurement limitations of the chosen approach and how to account for them.

The sample preparation included the addition of sorbitol as a plasticising agent to enhance film formation and handling. Post-processing steps were not utilized to keep the process as straightforward as possible for the purposes of this thesis. Mycelium -cellulose microfibril (CMF) composite films were used to study how a produced protein retained and bound in the casting suspension could directly affect material properties. Most of the films were pure mycelium (with sorbitol) since that was the material of interest. The casted films were measured for mechanical properties.

The mycelium cultivation, production, and post-production parameters were kept as constant as possible to facilitate drawing conclusions about the role of single genes. In addition to the mechanical tests, the different mycelium samples were characterized for growth, biochemical properties and filamental mycelium microstructure. This work provides a basis for further development of novel mycelium materials.

5 Materials and methods

The production of mycelium film material by the studied *T. reesei* strains involved a multistep process and various analysis methods to test material properties of the produced mycelium, that are disclosed in Scheme 1. The selected fungal strains, the production process, the analyses of this work and the related materials and methods are discussed in this chapter.



Scheme 1. Production of mycelium films and the determined properties for various products during the production. The production started from fungal spores (P1.) that were inoculated (1.) into *Trichoderma* minimal medium and cultivated for 5 d (2.) to obtain mycelium culture (P2.) (I.). Samples were taken from the mycelium culture to determine yield (A.), microstructure (B.), and protein expression (C.) patterns for the produced mycelium. The mycelium culture was filtrated (3.), washed (4.), and resuspended (5.) to obtain mycelium biomass (P3.) (II.). A sample was taken from the mycelium biomass to determine polysaccharide content (D.) for the mycelium. The mycelium biomass was formulated (6.) with sorbitol and in some cases with cellulose microfibrils, homogenised (7.) and degassed (8.) to obtain mycelium suspension (P4.) (III.). Mycelium suspension was cast (9.) in a temperature cabinet for 7 h at 70 °C to obtain mycelium film (P5.) (IV.). Physical properties (E.) and mechanical properties (F.) were determined from the mycelium film.

5.1 Selection of fungal strains

Fourteen strains of filamentous fungi *T. reesei* were chosen for this work to be cast as a mycelium film material with sorbitol acting as a humectant to promote leather-like properties via higher elasticity, which consecutively should make differences in the mechanical properties more apparent and comparable between fungal strains. The studied strains are listed in Table 2. The strains were designated based on either their genetic modifications or their protein production phenotypes in comparison to their parent strain. The six sample groups included genetically engineered strains with differences in expression of hydrophobin, resilin-like protein (RLP), spider silk protein fragment or chitinase enzyme. Five of the six sample groups utilized a pure mycelium-sorbitol mixture films while the sixth sample group investigated a composite of a mixture of mycelium and sorbitol mixture film reinforced with CMF. A mycelium-CMF composite film was used to study how the produced RLP could be retained and bound in the casting suspension and affect material properties.

Table 2. *T. reesei* strains used in the experiments.

Strain[a] gene/protein	Studied protein	Strain code	Genotype	References
QM9414	HFBI	VTT D-74075	reference	Mandels et al. (1972, 1971)
Δhfb1	HFBI	VTT D-99724	deletion of *hfb1*	Askolin et al. (2005)
hfb1+++	HFBI	VTT D-98692	multicopy of *hfb1*	Askolin et al. (2001)
Rut-C30	HFBII	VTT D-86271	reference	Montenecourt and Eveleigh (1979)
Δ*hfb2*	HFBII	VTT D-99676	deletion of *hfb2*	Askolin et al. (2005)
hfb2+++	HFBII	VTT D-99745	multicopy of *hfb2*	Bailey et al. (2002)
M658	RLP fusion	M658	reference	Landowski et al. (2013)
dCBM-RLP-HFBI	RLP fusion	M1230	gene for dCBM-RLP-HFBI	Griffo et al. (2017)
CBM-RLP-CBM	RLP fusion	M1438	gene for CBM-RLP-CBM	Fang et al. (2017)
M2027	silk fusion	M2027	reference	Valkonen, M., unpublished
AQ12	silk fusion	M2413	gene for AQ12	Westerholm-Parvinen, A., unpublished
CBM-AQ12-CBM	silk fusion	M2414	gene for CBM-AQ12-CBM	Westerholm-Parvinen, A., unpublished
M1909	chitinase	M1909	reference	Westerholm-Parvinen, A., unpublished
chitinase	chitinase	M1909-chit	gene for a chitinase	Valkonen M., unpublished

[a]The strains were named in this work based on their genotype and protein expression phenotype in comparison to their respective parent (reference) strain.

Hydrophobin strains

The first two sample groups studied the effect of overexpression and deletion of the *T. reesei hfb1* and *hfb2* hydrophobin genes, which code for the proteins HFBI and HFBII, respectively. These hydrophobin genes were first isolated by Nakari-Setälä et al. (1997, 1996). Hydrophobins are amphiphilic proteins that can assemble at hydrophilic-hydrophobic

interfaces and thus tend to aggregate on air-liquid interfaces (Nakari-Setälä et al., 1997; Wösten et al., 1993). Surface forces overcome gravitational forces at the microbial scale, emphasizing the importance of the ability of hydrophobins to lower surface tension and alter surface polarity by binding on the surface (Linder et al., 2005). Hydrophobins are categorized in two classes based on their hydropathy and biophysical patterns. Both HFBI and HFBII hydrophobin proteins are typically categorized as class II hydrophobins, which are secreted and found as soluble in the culture medium (Nakari-Setälä et al., 1996). HFBI is also found associated to the mycelium surface (Nakari-Setälä et al., 1996), while HFBII can also be found on the surface of spores (Nakari-Setälä et al., 1997).

The respective parent strains were used for reference samples: strain QM9414 for the HFBI sample group and strain Rut-C30 for the HFBII sample group. The high-cellulase producing mutant QM9414 had been originally generated by Mandels et al. (1972, 1971) utilizing a two-step irradiation process with a high-voltage linear electron accelerator by first irradiating conidia of the wild-type strain QM6a twice to obtain the QM9414 strain. The wild-type strain QM6a was originally thought to belong to *T. viride* sp., but it was later identified as a member of a completely new species named *T. reesei* (Peterson and Nevalainen, 2012; Simmons, 1977). Rut-C30 strain had originally been obtained by a three-phase mutagenesis form QM6a first by UV-irradiation, then chemically by nitrosoguanidine and finally again by UV-irradiation (Montenecourt and Eveleigh, 1979). Both QM9414 and Rut-C30 are nowadays considered as wild-type strains (Peterson and Nevalainen, 2012).

The deletion strains for the HFBI and HFBII sample groups were originally constructed by Askolin et al. (2005). The *hfb1* gene deletion strain Rut-C30 Δ*hfb1* was obtained by replacing the *hfb1* gene in the genome of the QM9414 strain with the *Aspergillus nidulans* acetamidase (*amdS*) gene as the selective knock-out marker. The *hfb2* gene deletion strain QM9414 Δ*hfb2* was obtained using an *Escherichia coli* hygromycin B phosphotransferase (*hph*) antibiotic resistance gene as the selective knock-out marker.

The multicopy strains for the HFBI and HFBII sample groups were originally constructed by two separate authors. The *hfb1* multicopy strain was constructed by Askolin et al. (2001) by co-transforming the strain QM9414 using the plasmid containing the *T. reesei hfb1* gene under the native glucose-inducible promoter and the plasmid containing the *amdS* gene as a

selective marker resulting in the QM9414 *hfb1+++* strain that contains three copies of the *hfb1* gene in its genome. The *hfb2* multicopy strain was constructed by Bailey et al. (2002) by incorporating the *T. reesei hfb2* gene under the native promoter for the *T. reesei* cellobiohydrolase gene *cbh1* in the genome of the strain Rut-C30 to obtain Rut-C30 *hfb2+++* strain that contains both the endogenous *hfb2* gene and three copies of the *hfb2* genes under the *cbh1* promoter.

Resilin-like protein producing strains

Two strains that produce slightly different fusion proteins were utilized to study the effect of RLPs on the mycelium. Both constructs contain a part of insect resilin protein which has a very high elasticity and is one of the most resilient materials known to humans. In the dCBM-RLP-HFBI construct the RLP from *Drosophila melanogaster* Rec1 resilin is flanked by a pair of cellulose binding modules (CBMs) originating from *T. reesei* cellobiohydrolases Cel6A and Cel7A, and the *T. reesei* HFBI In CBM-RLP-CBM, the resilin domain is flanked by the two CBMs (Fang et al., 2017; Griffo et al., 2017).

The parent strain M658 for the RLP constructs was used as a reference strain in the experiments and it had 10 disruptive protease genes deleted from its genome to potentially improve the production of heterologous therapeutic proteins (Landowski et al., 2013, 2015). The dCBM-RLP-HFBI producing strain was originally constructed by (Griffo et al., 2017) to enable RLP to act as an elastic connector material between the material components in hybrid nanomaterials. The CBM-RLP-CBM producing strain was originally constructed by (Fang et al., 2017) to investigate pH-responsive cellulose material coatings to potentially enhance material properties.

Spider silk producing strains

The recombinant *T. reesei* strains producing the silk protein fragment AQ12 were utilized to test whether production of spider silk would affect mycelium properties. Silk is a strong and tough protein fibre composed mainly of fibroin that is produced by many insects such as the mulberry silkworms and spiders (Mohammadi et al., 2019; Sutherland et al., 2010). The spider silk AQ12, also known as eADF3 (Batys et al., 2021), is an engineered version of the wild-type ADF3 dragline spidroin from *Araneus diadematus* spider (Mohammadi et al., 2019). The parent strain M2027 (Valkonen, M., unpublished) was used as the reference strain. Two

AQ12 silk protein producing strains were used, one secreting AQ12 alone and another secreting the fusion protein CBM-AQ12-CBM, where the silk fragment is flanked by two CBMs. Studies related to the material properties of both silk proteins produced by the unicellular *P. pastoris* (instead of the filamentous *T. reesei* used in this experiment) were conducted by Mohammadi et al. (2019, 2018b, 2018a).

Chitinase producing strain

Strains that produce cell wall degrading enzymes were utilized to study the role of the cell wall on the mycelium properties. As these enzymes are secreted through the cell wall, they might influence the cell wall composition and therefore these enzyme producing strains can potentially provide insight on factors contributing to mycelium properties. To test this idea, a native *T. reesei* chitinase overproducing strain was utilized. Chitinases are hydrolytic enzymes capable of breaking down glycosidic bonds within chitin polysaccharide (Patil et al., 2000). A chitinase overproducing strain, dubbed M1909 chitinase in this work, was used in the experiments and its parent M1909 was utilized as the reference strain (Valkonen, M., unpublished).

5.2 Production of mycelium films

The selected *T. reesei* strains were prepared for the inoculation of the cultivation medium. The spore suspensions (stored at -80 °C) of interest were thawed and plated on Potato Dextrose Agar (84651.0500) (VWR Chemicals, USA) plates and cultivated for 7 days at +28 °C. Sterile 0.8 % sodium chloride (NaCl) 0.025 % Tween 20 (Merck, Germany) water solution was added on the plate and the spores were re-suspended. The suspension was filtered through sterile cotton wool and the spore count was determined using LUNA-II (Thermo Fisher Scientific, USA) automated cell counter. For that, a 100-times dilution was prepared in 0.8 % NaCl 0.025 % Tween 20 water-based solution. These secondary stock spore suspensions were stored at -80 °C for inoculation.

5.2.1 Cultivation of fungal strains

Trichoderma minimal medium (TrMM) supplemented with a suitable carbon source was prepared as shown in Table 3. The promoter *cbh1* required lactose or other similarly inducing sugars for high protein expression, while the native *hfb1* promoter was induced in the

presence of glucose. Inoculation of between 50 000 and 100 000 spores were added (in a volume of between 5 and 100 µL) into 300 mL of TrMM in a 2 L Erlenmeyer shake flask capped with aluminium foil. The strains were cultivated for 5 days at +28 °C at 200 rpm similar to the method by Askolin et al. (2005), either in an open-room environment under regular electrical light utilizing an open-type Orbitron (INFORS HT, Switzerland) incubator shaker or in the dark enclosed in a New Brunswick Innova 44/44R (Eppendorf, Germany) incubator shaker.

Table 3. Recipe for 1000 mL of Trichoderma minimal medium.

Substance	Amount	Purpose
distilled de-ionized water (DDIW)	960 mL	solvent
potassium dihydrogen phosphate (KH_2PO_4)	15 g	element source
ammonium sulfate ($(NH_4)_2SO_4$)	5 g	element source
proteose peptone	2 g	nutrient source
ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)	5.0 mg	element source
manganese sulfate monohydrate ($MnSO4 \cdot H_2O$)	1.6 mg	element source
zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)	1.4 mg	element source
cobalt(II) chloride hexahydrate ($CoCl_2 \cdot 6H_2O$)	3.7 mg	element source
potassium hydroxide (KOH)	-	pH adjusting
calcium chloride ($CaCl_2$), 1 M	4.1 mL	salts
magnesium sulfate ($MgSO_4$), 1 M	2.4 mL	salts
glucose or lactose	40 g	carbon source

5.2.2 Preparation of mycelium films

To harvest the mycelium, the *T. reesei* culture was vacuum filtered utilizing DOA-P730-BN (PALL Life Sciences, USA) vacuum/pressure pump through Whatman grade GF/B glass microfibre filters (Cytiva, USA) in batches of around 800 mL. Each of these batches were washed with three subsequent additions of 100 mL of distilled de-ionized water (DDIW) and the mycelium biomass layer remaining on the filter was collected. The mycelium was re-suspended in between 5 and 20 mL of DDIW depending on the rough amount of mycelium obtained. The dry weight content was determined as discussed below in section 5.3. The following day, the mycelium biomass was resuspended in DDIW to obtain a 6 % (w/v) mycelium suspension based on the mycelium yield.

Sorbitol was added in the ratio 1:4 of sorbitol to mycelium as a humectant. A CMF water solution was prepared for the casting suspensions for mycelium-CMF composite films. The Celish PC110S filtration grade (Daicel, Japan) CMF was added into DDIW and mixed with a

HR1360 series (Philips, Netherlands) hand blender for three subsequent 5 min intervals at full power to obtain a 4 % (w/v) CMF water solution. The mycelium to CMF to sorbitol ratio in the casting solution was 3:1:1. DDIW was added to obtain a dry substance content of 5.4 % comprising both mycelium and sorbitol, in the casting suspension. For the mycelium-CMF composite films, the casting suspensions were prepared so that the total dry substance content – formed by mycelium, sorbitol, and CMF – was 5.4 %.

The casting suspensions were homogenized with a Polytron PT 6100 (Kinematica, Switzerland) homogenizer using a Polytron Generator PT-DA 20/2EC-B193 (Kinematica, Switzerland) shearing aggregate for 5 min at 10 000 rpm. The homogenized biomass was degassed with SpeedMixer DAC 1100.1 VAC-P (Synergy Devices Limited, UK) vacuum mixer system for 5 min at 1600 rpm at around -0.7 bar vacuum pressure.

Approximately 100 g of the degassed mycelium suspensions was cast on a plastic petri dish with an area of 600 cm^2 for all the other sample groups except for the HFBI sample group, which had approximately 40 g of the degassed casting suspensions cast on a plastic petri dish with an area of 240 cm^2 due to the low mycelium yield. Each petri dish was then put into a B8133 (Termaks, Norway) temperature cabinet for 7 h at +70 °C for drying to obtain dry mycelium films.

5.3 Mycelium yield analysis

Mycelium yield was used to determine both the mycelium production capability and the formulation for mycelium films.

A sample of between 25 and 50 mL was taken from each cultivation medium right after the cultivation was stopped for determination of mycelium yield. The sample was vacuum filtered utilizing the DOA-P730-BN (PALL Life Sciences, USA) vacuum/pressure pump through a Whatman grade GF/B glass microfibre filter (Cytiva, USA). The filter was dried in an oven at +100 °C overnight, and the mycelium yield was determined by weighing (Sartorius, Germany) the filter paper.

5.4 Microstructural analysis

Optical microscopy was utilized to determine the microstructure of the mycelia produced by the studied strains (Figure 3). Changes in microstructure could potentially predict changes in material properties tested later in this work.

All microscopy samples were taken right before the cultivation medium was filtered. 1:99 dilutions were prepared from the 500 µL samples with DDIW. Polyvar (Reichert, Austria) microscope with a 40x Plan (Reichert, Austria) achromatic bright-field objective lens was used for microscopy imaging of the samples. The images were captured using Leica Application Suite Version 2.8.1 (Leica, Germany) imaging software with a camera (Leica, Germany) attached to the microscope. For this work, microstructural quantitation was not done.

5.5 Protein expression analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analyses were used to confirm whether a specific protein was produced or absent as intended for the specific engineered strains.

Samples of 500 µL were collected from each cultivation right before harvesting the mycelium by filtration. The samples were centrifuged for 5 min at 21 000 g with a microcentrifuge (Eppendorf, Germany). The supernatant was collected and 500 µl of DDIW was added to the pellet fraction for washing and the mycelium pellet was centrifuged again for 5 min at 21 000 g. The supernatant for washing was discarded and the mycelium pellet was collected. Both the collected supernatant and the mycelium pellet samples were stored at -20 °C for up to 4 months.

The pellet and supernatant culture samples were thawed and prepared for SDS-PAGE as follows. The 4-times concentrated Laemmli Sample Buffer (LSB) (4x LSB) stock solution – containing Tris-HCl, glycerol, SDS, Orange G dye, β-mercaptoethanol and DDIW – was diluted to 1-time concentrated LSB (1x LSB) solution and 125 µL of this solution was added to each pellet sample and resuspended with a vortex mixer. The pellet samples of the cultivations were incubated for between 1 and 2 h at room temperature and mixed with a vortex mixer every 10 to 30 min. Excessive mixing was avoided to prevent the releasing of DNA or other

potentially undesired disruptive material from within the cells making up the mycelium pellets. After the incubation period, the pellet samples were centrifuged for 5 min at 21 000 g, and the supernatant fraction was collected.

The culture supernatant samples for SDS-PAGE were prepared by mixing 20 µL of 4x LSB with 60 µL of the sample. Both the pellet and the supernatant samples were then boiled for either 5 or 10 minutes at 100 °C using a heater block, then centrifuged for 5 min at 21 000 g and the supernatants were collected. The Precision Plus Protein All Blue Precision Standards (Bio-Rad, USA) solution was utilized as the molecular weight marker (MWM). The samples were gently spun down with a minicentrifuge, and 5-15 µL (without the volume of LSB) of the samples were loaded onto 4-20 % gradient Criterion TGX gels (Bio-Rad, USA). The running buffer was Tris-Glycine-SDS. The SDS-PAGE run was done using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, USA) for 30 to 60 min at 200 V.

For SDS-PAGE Coomassie analysis, the rinsed SDS-PAGE gels were stained with 20 – 50 mL PageBlue Protein Staining Solution (Thermo Fisher Scientific, Lithuania) and incubated overnight on a horizontal shaker at 50 rpm at room temperature. The gels were then de-stained by washing repeatedly with Milli-Q water. The gels were imaged with Molecular Imager Gel Doc XR+ Imaging System (Bio-Rad, USA) using Image Lab Version 5.1 (Bio-Rad, USA) image capturing and processing software.

For western blotting, the proteins on the SDS-PAGE gels were blotted onto nitrocellulose membranes using a Trans-Blot Turbo Transfer System (Bio-Rad, USA) and Trans-Blot Turbo Transfer Pack (Bio-Rad, USA). Tris-buffered saline (TBS) Tween (TBST) solution was prepared from a 10-times concentrated TBS (10x TBS) stock solution. 5 % (w/w) milk protein powder TBST suspension was prepared by adding 5 g of the Skimmed Milk Powder (Valio, Finland) to 95 g of TBST. This milk protein TBST suspension was used to block the empty areas in the nitrocellulose membrane by incubating at 4 °C overnight on a horizontal shaker. The membranes were rinsed with TBST, and 20 mL of a 1:2000 diluted primary antibody TBST solution was added, followed by incubation at 50 rpm shaking for 1 h at room temperature.

For detecting HFBI or HFBII proteins the non-commercial rabbit polyclonal antibodies anti-HFBI, and anti-HFBII, respectively, were used. To detect proteins containing a flag-tag (dCBM-

RLP-HFBI, CBM-RLP-CBM, AQ12 and CBM-AQ12-CBM) the mouse polyclonal StrepMAB-Classic (2-1507-001) (IBA, Germany) antibody was used. After the primary antibody incubation, the membranes were rinsed with TBST, and 20 mL of a specific 1:30 000 diluted secondary IR-dye conjugated antibody in TBST was added and incubated shaking at room temperature for 1 h to detect the target proteins.

As the secondary antibody the IRDye 680RD Goat anti-Rabbit Immunoglobulin G (LI-COR, USA) was used for detection of the HFBI and HFBII proteins, and IRDye 680RD Goat anti-Mouse Immunoglobulin G (LI-COR, USA) for the detection of flag-tag containing proteins. Finally, the blots were rinsed with TBS and imaged with an Odyssey CLx (LI-COR, USA) scanner at 700 nm using Image Studio Version 5.2 (LI-COR, USA) image processing software.

5.6 Physical and mechanical determination

Material properties of mycelium films were investigated by determining various physical and mechanical properties. The main physical property of the film determined was density, while the main mechanical properties were Young's modulus, ultimate tensile strength (UTS) and strain at UTS. The same film samples were used to first determine the weight and area of a film, followed by the slicing of that film for the determination of thickness and mechanical properties. The thickness in combination with the known area and the mass of the cast film was measured (data not shown) to determine the density of each film.

The dry films were taken into a standard condition room at 23 °C in 50 % relative humidity. Each film was let to stabilize for at least a week and up to two months before mechanical testing. The mycelium film tensile measurements were based on the ISO 3376:2020 standard for determination of tensile strength and percentage elongation for leather material (International Organization for Standardization, 2020) with adjustments and deviations fitting for the produced mycelium bioleather material for practicality reasons. The procedure is as follows.

Six 10 mm (± 1 mm) wide and 60 mm (± 5 mm) long rectangular slices were cut from the cast mycelium films. Film thickness was measured as an average using a Lorentzen & Wettre thickness gauge from three points for each film slice. A balance (Precisa, Switzerland) was

used to determine weight for the films. Thickness, surface area and weight of the films were used to determine density of the films. The LS5 (Lloyd Instruments, UK) materials testing machine and the YLC 100N (Ametek, USA) load cell were used to determine Young's modulus, UTS and strain at UTS for between two and six parallel samples depending on the sufficiency of the film slices. A uniform separation speed of 21 mm/min was utilized and the jaws of the tensile tester were 50 mm apart. The measurements were collected and processed with NEXYGEN Plus 3.0 (Lloyd Instruments, UK) software. A conventional interquartile range (IQR) method (Dekking et al., 2005) was used as the measure of the statistical dispersion for the thickness, Young's modulus, UTS and strain at UTS for each mycelium film.

5.7 Fungal cell wall polysaccharide characterization

The polysaccharide content of the mycelium suspension was analysed to better understand whether the genetic engineering of the strains had affected the structure and composition of the cell wall. A method derived from previously published work (Mohammadi et al., 2012; Zamani et al., 2008; François, 2007) was established in collaboration with researchers and technicians at VTT for measuring the cell wall polysaccharide content. The method was based on chemical hydrolysis of the cell wall saccharides to monomer form which were then identified and quantified using liquid chromatography.

Samples (5 mL) for the analysis were taken from the 6 % (w/v) mycelium suspensions, stored at -20 °C. The samples were placed into a freezer at -80 °C for between 1 and 2 h and they were freeze-dried using an Alpha 2-4 LSCbasic (Martin Christ, Germany) freeze-dryer system first for 24 h at 0.5 mbar and then 45 h at 0.1 mbar. In freeze-drying, the water is removed as ice from the object via sublimation in a low pressure or vacuum (Prosapio and Lopez-Quiroga, 2020). One round metallic bullet having a diameter of 10 mm and two round metallic bullets having a diameter of 5 mm were added to the dried mycelium before grinding. The dried samples were ground using a MM 301 (Retsch, Germany) mixer mill for 2 min at 30 Hz. The ground mycelium samples were washed three times with 1 ml of DDIW and centrifuged in between for 5 min at 4000 g. The mycelium pellet fractions, which contained the cell wall material, were collected, and the supernatant fractions for washing were discarded each time. The washed mycelium cell wall pellets were re-suspended in 1 ml of DDIW and mixed with a vortex mixer to obtain homogenous suspensions that were stored at

-20 °C. The samples were placed into a freezer at -80 °C for 1-2 h and the freeze-drying step was repeated for 23 h at 0.5 mbar. The freeze-dried mycelium cell wall samples were then ground using the same parameters and equipment as before. The ground mycelium cell wall samples were stored in an exicator cabinet until further processing.

The samples for cell wall polysaccharide content analysis were hydrolysed in four separate batches using Rut-C30 as the reference sample for the hydrolysis treatments and the liquid chromatography analysis. Each pre-treated sample was dissolved as 10 mg in 150 µL of 72 % (v/v) sulfuric acid (H_2SO_4) water solution, mixed with a glass stirring rod and left to incubate for 2 h for the cell wall hydrolysis. To each sample 1.90 mL ultrapure water was added. The samples were incubated for 4 h at +100 °C in a water bath in a temperature cabinet (WTB Binder, Germany). Then 10 ml of ultrapure water was added to the samples. The pH of each sample was adjusted between pH 6 and pH 8 with 8 to 12 mL of 0.2 M saturated barium hydroxide ($Ba(OH)_2$) (Merck, Germany) water solution, and they were kept at +4 °C overnight. The next day, the pH value was checked again and adjusted in the range of pH 6 and pH 8 with a few drops of either 0.1 M H_2SO_4 or 0.2 M $Ba(OH)_2$ (Merck, Germany). The total sample volume for each tube was adjusted to 30 mL with DDIW and the samples were centrifuged for 15 min at 3200 g at +4 °C. A 1 mL volume of each sample supernatant was collected and centrifuged for 5 min at 8000 g and a 200 µl volume of this supernatant was pipetted on a 96-well microplate and stored at -20 °C until the analysis.

For analysing the cell wall hydrolysates a Dionex ICS-6000 (Thermo Fisher Scientific, USA) high pressure ion chromatography (HPIC) running the Chromeleon (Thermo Fisher Scientific, USA) processing software was used. A Dionex CarboPac SA10-4µm (2 × 250 mm, particle size 4 µm) (Thermo Fisher Scientific, USA) was used as the column with a Dionex CarboPac SA10-4µm Guard Column (2 × 50 mm) (Thermo Fisher Scientific, USA) and an electrochemical (Gold, Carbo, Quad) pulsed amperometric detector. The column temperature was set to 40 °C. The sample injection volume was 2.5 µl, the eluent was 12 mM KOH, and the flow rate was 0.38 mL/min. Glucose, galactose, mannose, and glucosamine were used as the monosaccharide standards to calibrate the HPIC system.

Since the exact cell wall composition is still unknown certain assumptions about the polysaccharides had to be made. For determining the cell wall polysaccharide content, the

detected glucosamine in the hydrolysates was assumed to originate from cell wall chitin, the detected glucose from glucans, and the detected galactose and mannose from galactomannans. Free intracellular monosaccharides were removed during sample preparation by a wash step in order to avoid interference with the analysis.

When a glycosidic bond is formed between two monomers, a water molecule is lost in the condensation reaction (Brock et al., 2003). This needs to be accounted for when calculating the polysaccharide quantity from their monomers. It can be reasonably assumed that there are relatively few open ends in the polysaccharide chains as compared to the number of monomers in the polymers. Therefore, the determination of mass for a specific polysaccharide could be conducted by subtracting the molar mass of a water molecule from the molar mass of the corresponding monosaccharide (yielding the molar mass of the monomer unit) and multiplying that sum with the determined corresponding total monosaccharide amount of substance measured by the HPIC analysis, as can be seen in equation (1).

$$m_{PS} = M_{MU} \cdot n_{MS} = (M_{MS} - M_W) \cdot n_{MS} \quad (1)$$

where m_{PS} is mass of a polysaccharide (g)

M_{MU} molar mass of a monomer unit (g/mol)

n_{MS} amount of substance of a monosaccharide (mol)

M_{MS} molar mass of a monosaccharide (g/mol)

M_W molar mass of water (g/mol)

The deacetylation ratio of the chitin changes the average weight of each monomer unit in the polysaccharide chain and should be considered in the analysis, which was accomplished with equation (2). As the hydrolysis step of the sample preparation converts the acetylated N-acetylglucosamine monomer into deacetylated glucosamine the analysis method cannot distinguish between the two monomer forms. Furthermore, literature values for the de-acetylation of chitin were scarce, but it has been claimed that the amount of chitosan in *T. reesei* is relatively low (Kappel and Gruber, 2020). For these reasons, in this work, an average acetylation ratio of 75 % for chitin was assumed.

$$M_{MU} = M_{MS} - M_W = r_1 \cdot M_{MU1} + r_2 \cdot M_{MU2} - M_W = r_1 \cdot M_{MU1} + (1 - r_1) \cdot M_{MU2} - M_W \quad (2)$$

where M_{MU} is molar mass of a monomer unit (g/mol)

M_{MS} molar mass of a monosaccharide (g/mol)

M_W molar mass of water (g/mol)

r_1 acetylation ratio (%)

r_2 deacetylation ratio (%)

M_{MS1} molar mass of an acetylated monosaccharide (g/mol)

M_{MS2} molar mass of a deacetylated monosaccharide (g/mol)

6 Results

All the *T. reesei* strains in this study were examined at the biochemical, microscopical and macroscopical level to study the effect of single gene expression on the material properties of mycelium. In this chapter, the experimental results are reported.

6.1 Mycelium yield

The mycelium yields from the cultivations using the studied strains are shown in Table 4. The yields were in the range of 2.7 and 4.7 g/L, except for the chitinase strains which produced a low amount of mycelium between 0.4 and 2.3 g/L.

Table 4. Mycelium yields for the studied *T. reesei* strain cultivations.

Strain gene/protein	Studied protein	Fibres in film[a]	Carbon source	Cultivation condition	Mycelium yield[b] (g/L)	Mycelium yield in relation to reference
QM9414	HFBI	-	glucose	light, room	3.2	-
Δ*hfb1*	HFBI	-	glucose	light, room	3.3	103 %
hfb1+++	HFBI	-	glucose	light, room	3.2	100 %
Rut-C30	HFBII	-	lactose	light, room	4.1	-
Δ*hfb2*	HFBII	-	lactose	light, room	4.3	105 %
hfb2+++	HFBII	-	lactose	light, room	4.7	115 %
M658	RLP fusion	-	lactose	dark, incubator	3.2	-
dCBM-RLP-HFBI	RLP fusion	-	lactose	dark, incubator	3.1	98 %
CBM-RLP-CBM	RLP fusion	-	lactose	dark, incubator	3.1	99 %
M658	RLP fusion	CMF	lactose	dark, incubator	3.2	-
dCBM-RLP-HFBI	RLP fusion	CMF	lactose	dark, incubator	3.1	97 %
M2027 (a cultivation)	silk	-	lactose	dark, incubator	2.9	-
AQ12	silk	-	lactose	dark, incubator	2.8	102 %
M2027 (b cultivation)	silk fusion	-	lactose	dark, incubator	2.7	-
CBM-AQ12-CBM	silk fusion	-	lactose	dark, incubator	2.8	100 %
M1909	chitinase	-	lactose	dark, incubator	0.4	-
M1909 chitinase	chitinase	-	lactose	dark, incubator	2.3	563 %

[a]The fibres were added to the mycelium film casting suspension after the cultivations. [b]The yield was determined for each strain from one sample only, so no standard deviation nor p-value (with Student's t-test) could be determined.

The mycelium yields within each sample group were remarkably equal except for the chitinase sample group. The repeated test cultivations for M1909 and M1909 chitinase strains confirmed the low yields (0.5 g/L and 1.8 g/L, respectively).

It was noticed during test cultivations that the open light room condition decreased the mycelium yield level in comparison to the dark environment provided by enclosed incubators, and that's why dark incubators where the preferred cultivation environment. It is possible that lactose as a carbon source reduced mycelium yields.

6.2 Microstructure of mycelium

The filamentous and branching nature of the mycelium was evident, and the hyphae thickness was similar for all strains, as can be seen in Figure 3. All the parent strains had very similar microstructures to the strains derived from them. M1909 chitinase strain had a more swollen bead-like structure than its parent strain M1909. Microstructural differences were more notable across all the sample groups differentiated by multiple genes instead of a single gene or a few genes within sample groups. Strains Rut-C30 and QM9414 and the strains derived from these had relatively long hyphae with few branching points. Whereas the genetically modified parent strains M658, M2027 and M1909 had short and hyperbranching hyphae with bead-like phenotype.

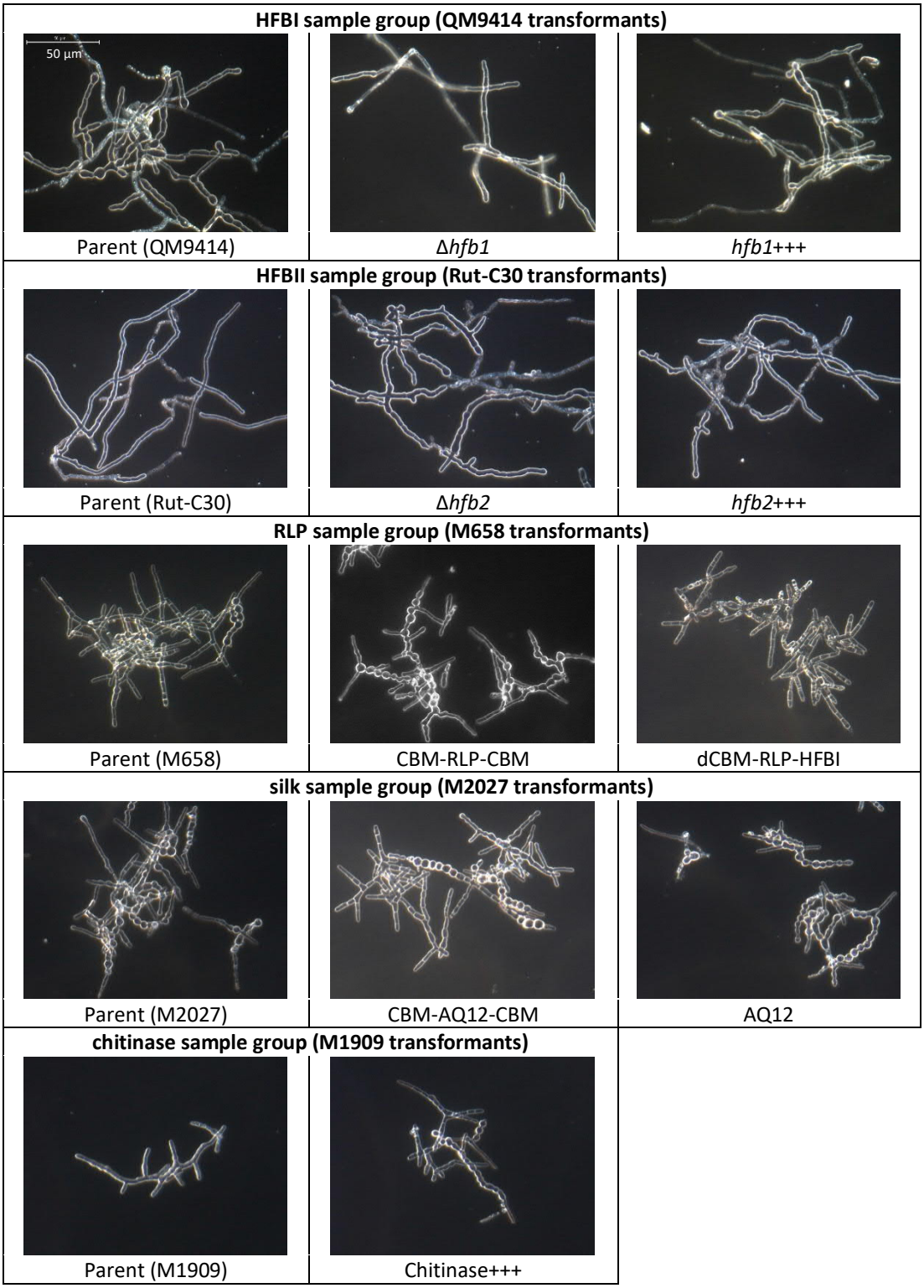


Figure 3. Bright-field microscopy images of the studied *T. reesei* strains. The parent strains for the genetically modified strains were used as reference samples within sample groups. Slight colour variations were caused by the automatic exposure adjustments in the equipment. The scale bar (50 µm) is shown in the image on the top left.

6.3 Protein expression in mycelium

The protein expression patterns of the studied strains were studied using SDS-PAGE and western blotting methods and the results were shown in Figure 4 for HFBI and HFBII and RLP sample groups, and in Figure 5 for the RLP (+CMF), silk and chitinase sample groups. The sample amount for analysis was equal in volume for all strains and was not adjusted to the mycelium yield. The chitinase producing strain and its parent strain had consistently very different mycelium yields, while the mycelium yields within each sample group for the rest of the strains were similar and comparable (Table 4), as was established before.

The cultivation media carbon source was chosen so that each promoter of the introduced genes would be active. However, the strongest induction with insoluble substrates was not used as any undigested insoluble particles would disturb the material analysis. Therefore, lactose and glucose were used as a carbon source.

The HFBI and HFBII protein productions were confirmed by western blotting (Figure 4A, 4B). Hydrophobins are small proteins with the size of about 7 kDa, but it has been observed before (Szilvay, G., personal communication) that they do not usually run true to size in SDS-PAGE. Therefore, visible bands below 10 kDa were identified as hydrophobins. The highest amount of HFBI was detected in the mycelium pellet fraction of the culture for the multicopy strain QM9414 *hfb1+++*, while some HFBI was detected for the reference strain QM9414. HFBI production was not detected for the culture of the *hfb1* deletion strain nor in any culture supernatant fractions in the HFBI sample group.

Similar results were obtained for the HFBII reference, deletion, and overproduction strains: a band corresponding to HFBII was observed in the mycelium pellet fraction for both the reference strain Rut-C30 and for the *hfb2* multicopy strain cultures. A faint HFBII band was also detected in the supernatant fraction for the *hfb2* multicopy strain culture. HFBII bands were not detected in the *hfb2* deletion strain culture.

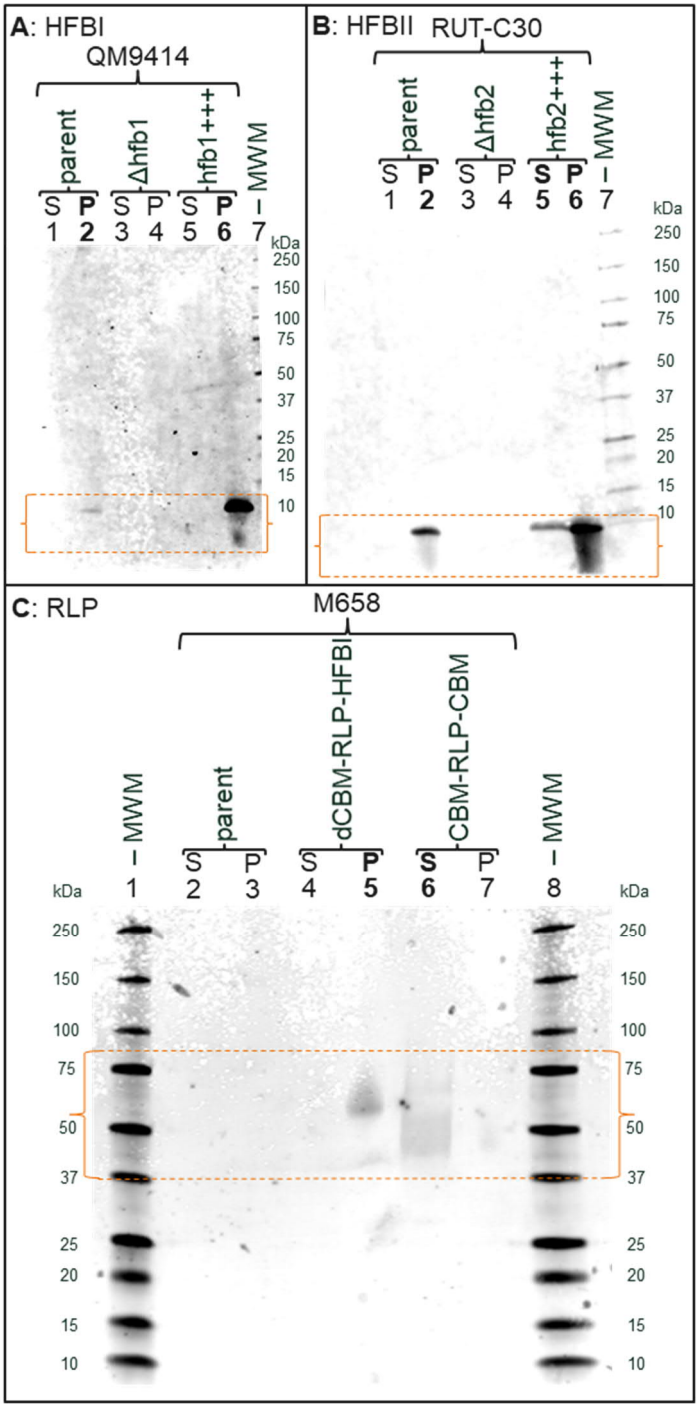


Figure 4. Western blots showing the target protein production for HFBI (A), HFBII (B), and RLP (C) sample groups divided to culture supernatant (S) and culture pellet (P) fractions. The proteins were detected using antibodies raised against HFBI (A), HFBII (B), and Strep-tag (C) as described in Materials and methods of this work. Sample volume was 15 µL for all QM9414-derived and Rut-C30-derived strains while the sample volume for M658-derived strains was 5 µL. The molecular weight marker (MWM) was added in 10 µL volume in all cases. The area of interest for specific target proteins for each sample group is denoted with the orange outlines. The lane number and the fraction designation (S or P) are bolded in case the protein of interest has been detected in that sample.

The RLP production was studied using both SDS-PAGE and western blotting (Figure 4C). Two separate cultivations of the RLP producing strains were performed: one for preparing a pure mycelium film and another for preparing a mycelium-CMF composite film. The studied RLPs had calculated sizes of 39 kDa (CBM-RLP-CBM) and 48 kDa (dCBM-RLP-HFBI) and due to glycosylations and possible proteolytic degradation the proteins were expected to run between 50 and 75 kDa in the electrophoresis (Szilvay, G., unpublished). The analysis by SDS-PAGE was complicated by many other highly expressed proteins that had similar sizes than the RLPs such as some cellulase enzymes and the results are therefore not shown.

The cultures used for preparing mycelium for pure mycelium films from RLP strains were analysed by western blotting. The results showed that while the parent strain M658 did not produce any detectable RLP fusion protein, both M658 dCBM-RLP-HFBI and M658 CBM-RLP-CBM strains had very low RLP production levels. Protein dCBM-RLP-HFBI was observed only in the mycelium pellet fraction and CBM-RLP-CBM protein only in the supernatant fraction.

Mycelium produced by M658 dCBM-RLP-HFBI was chosen to be combined with CMF in a later cultivation, since dCBM-RLP-HFBI was identified to be located in the culture pellet fraction indicating that dCBM-RLP-HFBI could be retained in the mycelium biomass even after washing and resuspending.

When the cultures used for preparing the mycelium-CMF composite films were investigated, RLP bands were not observed in the western blots for any of the RLP strains (data not shown). This was most probably due to an unknown technical reason. A band of corresponding size was observed in the SDS-PAGE gel (Figure 5A) in the mycelium pellet sample of M658 dCBM-RLP-HFBI, while other samples showed multiple unidentified bands. The results therefore remained inconclusive for the RLP production for the mycelium-CMF composite films.

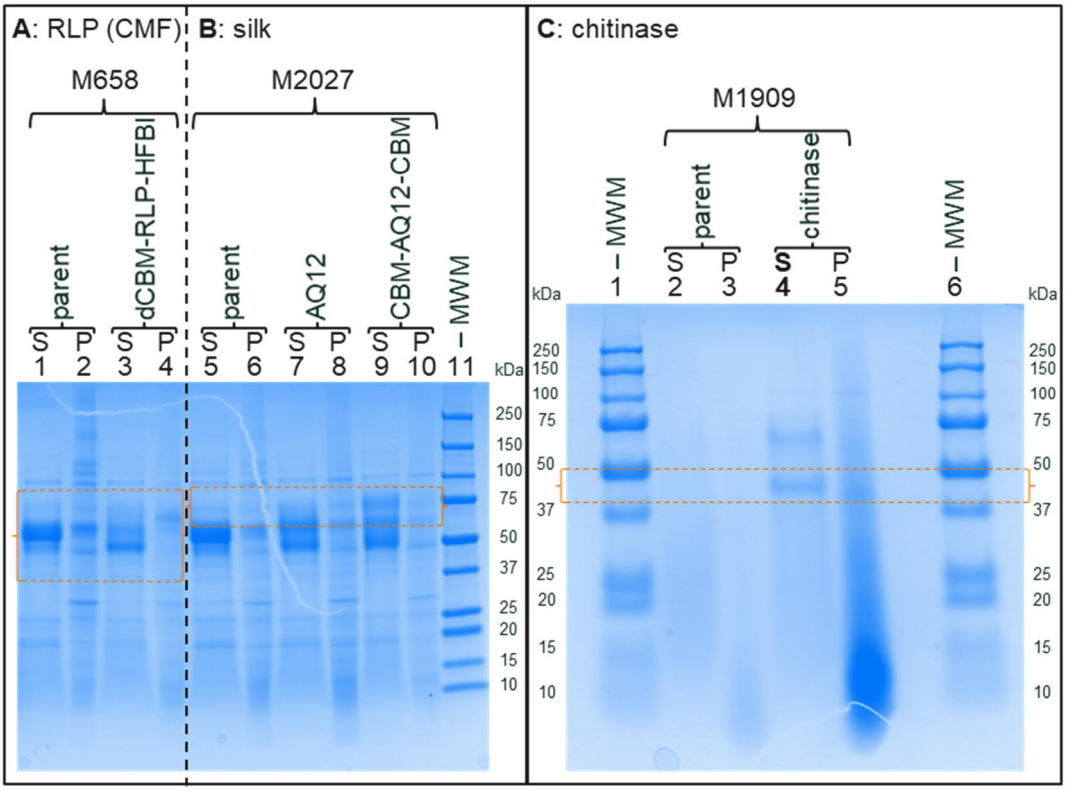


Figure 5. SDS-PAGE analysis of culture supernatant (S) and pellet (P) from RLP (to be cast with CMF) (A), silk fusion proteins (B) and chitinase (C) producing and their reference strains. The RLP producing cultures and their reference used for mycelium-CMF composite films were under investigation in this analysis. Sample volumes of 15 µL were utilized for all samples and 10 µL for MWM. The expected location for the proteins of interest for each sample group is marked with orange outlines. The sample number and the fraction designation (S or P) are bolded when the protein of interest has been possibly detected in that sample.

Results of western blots for culture samples of silk producing strains were not obtained, as the samples were on the same unsuccessful blot for the RLP strain samples (for CMF experiments) mentioned above. Therefore, the analysis was done by SDS-PAGE. Comparing the samples of the silk fusion protein producing strains to the parent strain showed slightly stronger bands at or below 75 kDa for the supernatant samples of M2027 AQ12 (calculated size 47 kDa) and M2027 CBM-AQ12-CBM (calculated size 58 kDa) (Figure 5B) indicating a possible silk fusion protein production. The studied silk fusion proteins typically run larger than their calculated size in SDS-PAGE (Szilvay, G., unpublished). Nevertheless, it remained inconclusive whether the strains were producing silk fusion proteins in the conditions applied in this study.

To study the expression of chitinase producing strains western blotting was not possible as antibodies against the enzyme were not available. Therefore, only SDS-PAGE was used to study the chitinase expression for the chitinase sample group (Figure 5C). In the supernatant of M1909 chitinase strain culture a band at around 45 kDa was visible indicating secretion of the studied chitinase with a calculated size of 43 kDa (Valkonen, M., unpublished). Native cellulases are also secreted in lactose conditions (e.g. via induction of the gene *cbh1*) and can typically be seen in the gel at around 75 kDa (Szilvay, G., personal communication). However, these bands were not visible in the parent strain sample. The data indicates that chitinase is being secreted by the M1909 chitinase strain in the used conditions, but a more direct identification method using antibodies would be needed to verify this finding.

6.4 Physical and mechanical properties of mycelium films

The determinations for density, mass, and area of the film utilized only one measurement point, while originally six film slices were cut from each mycelium film for the determination of thickness and mechanical properties. The results were then screened with the IQR method, and the number accepted of measurement points for thickness and mechanical measurements are shown in Table 5 and in Figure 6 and 7. As a very crude rule of thumb, at least five measurements could be considered as reliable for determination of a property. Approximately half of the mycelium films had at least five acceptable measurement points for thickness and mechanical property determination, while two samples had only two measurement points: QM9414 and QM9414 hfb1+++. The mechanical test results where the number of replicates was less than five should be considered unreliable. Those results were nevertheless included in this work as one of the aims of the study was to understand the limitation of the research approach.

6.4.1 Physical properties

Density, thickness, and area values for the mycelium films cast from the studied *T. reesei* strains, and the number of points for thickness measurements are shown in Table 5. Density described how tightly backed the mycelium was in the film. The average film density of all the sample groups was 290 g/cm^3 with a standard deviation of 40 g/cm^3 (relative: 14 %). The highest relative standard deviation for density within any sample group was 11 %. These indicated that differences in densities were not remarkable within the sample groups nor

between the sample groups. Interestingly, the density within the HFBII sample group was the lowest for the hydrophobin overexpression strain and the highest for the hydrophobin deletion strain. This finding is in line with a previous publication on mycelium and hydrophobin expression (Appels et al., 2018). However, the same relationship between the hydrophobin expression level and density was not as consistent for the HFBI sample group and the density values were mildly decreased for both the deletion and the overexpression strain of *hfb1*.

Table 5. Physical properties and the number of accepted parallel samples for the studied *T. reesei* mycelium films, and the dry content of the casting suspension.

Strain gene/protein	Studied protein (fibre)	Film density (kg/m³)	Film area (cm²)	Film thickness (µm)	p-value for thickness[a]	Measurement points[b] for thickness
QM9414	HFBI	270	240[c]	313 ± 15	-	3[d]
Δ*hfb1*	HFBI	260	240[c]	330 ± 8	0.1893	4[d]
hfb1+++	HFBI	260	240[c]	310 ± 14	0.8222	2[d]
Rut-C30	HFBII	240	600	362 ± 35	-	6
Δ*hfb2*	HFBII	270	600	328 ± 28	0.1084	5
hfb2+++	HFBII	220	600	**417 ± 36**	**0.0222**	6
M658	RLP fusion	350	600	251 ± 18	-	6
dCBM-RLP-HFBI	RLP fusion	310	600	277 ± 28	0.1569	4[d]
CBM-RLP-CBM	RLP fusion	300	600	**295 ± 10**	**0.0008**	4[d]
M658	RLP fusion (CMF)	250	600	352 ± 7	-	6
dCBM-RLP-HFBI	RLP fusion (CMF)	280	600	**327 ± 6**	**0.0001**	5
M2027	silk fusion	360	600	221 ± 33	-	6
AQ12	silk fusion	340	600	229 ± 6	0.5787	5
M2027	silk fusion	330	600	211 ± 18	-	6
CBM-AQ12-CBM	silk fusion	280	600	**273 ± 1**	**0.0003**	4[d]
M1909[e]	chitinase	-	-	-	-	-
chitinase	chitinase	310	600	277 ± 6	-	4[d]

[a]The p-value for thickness of each sample was determined using two-sample Student's t-test comparing each sample with its reference assuming unequal variance, normality, and independent and individually distributed data. The significance level (α) at 0.05 was chosen and p-values below that have been bolded along with their respective thickness values. [b]Measurement points for thickness denote the number of film slices taken from the same film for the thickness measurement. Film density, mass, and area had only one measurement and thus standard deviation could not be determined for them. [c]Mycelium film area was smaller for HFBI producing strains due to low mycelium yields. [d]For reliable results, five film slices would be preferred, so each sample not having at least five measurement points are marked. [e]Mycelium yield for the chitinase reference strain M1909 was too low for obtaining the needed biomass in a practical way for film casting.

Some of the film thicknesses had a low p-value associated with them implying a significant result. However, since thickness differences were partly caused by variance in casting suspension weights (data not shown), it was not strictly a universally comparable property of the mycelium films and was mainly a quality monitoring property for the film casting process,

while density was strictly a material property. Differences in thickness values could still reflect to other physical and mechanical values for the films.

6.4.2 Mechanical properties

The UTS and the tensile strain at UTS values for the produced mycelium films are shown in Figure 6 and the values for Young's modulus in Figure 7. UTS was between 1.4 and 7.6 MPa, Strain at UTS between 2 and 16 %, and Young's modulus between 41 and 520 MPa for all fungal strains. These values indicated that the variability between all samples was relatively high for all mechanical property determinations.

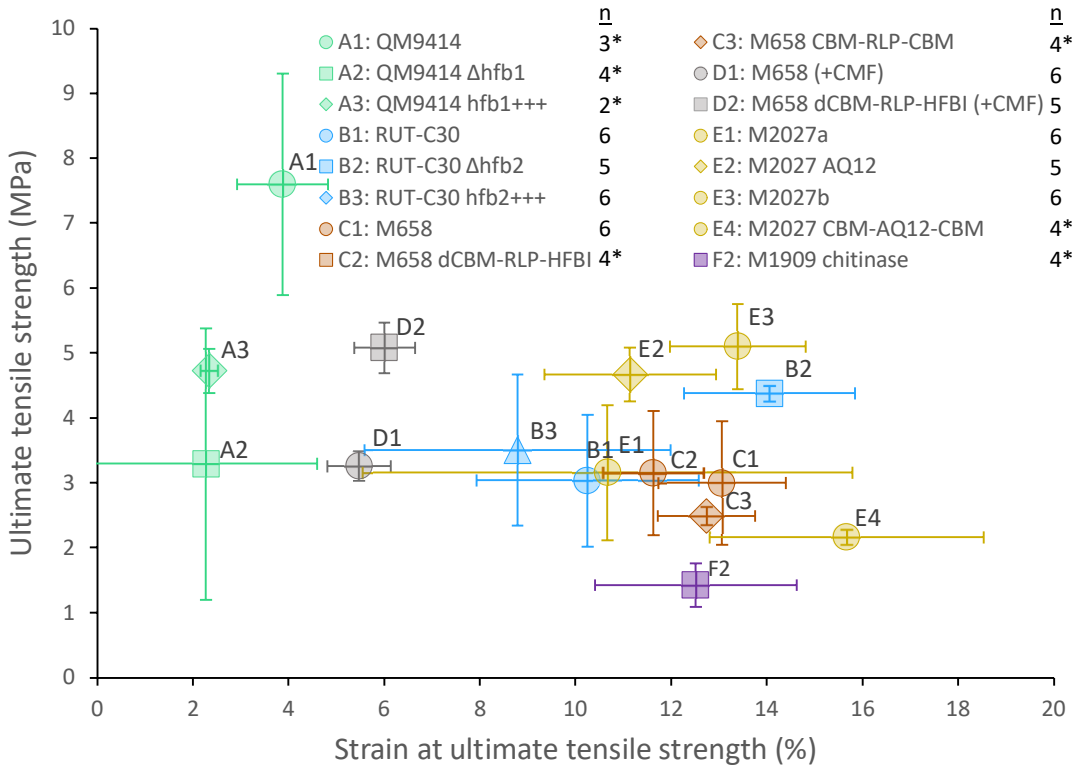


Figure 6. Ultimate tensile strength and tensile strain at ultimate tensile strength of the mycelium films for the studied *T. reesei* strains. Mycelium film samples are denoted with a circle for the reference fungal strains, with a square for the deletion strains or the first compared fungal strains and with a diamond shape for the over-expression strains or the second compared fungal strains. M2027a was the reference for the mycelium film produced by M2027 AQ12, and M2027b was the reference for the mycelium film produced by M2027 CBM-AQ12-CBM. Samples D1 and D2 were mycelium CMF composite films. The number of measure points (n) for each sample film has been marked next to the legends, and they range from two to six. *Four or less measurement points.

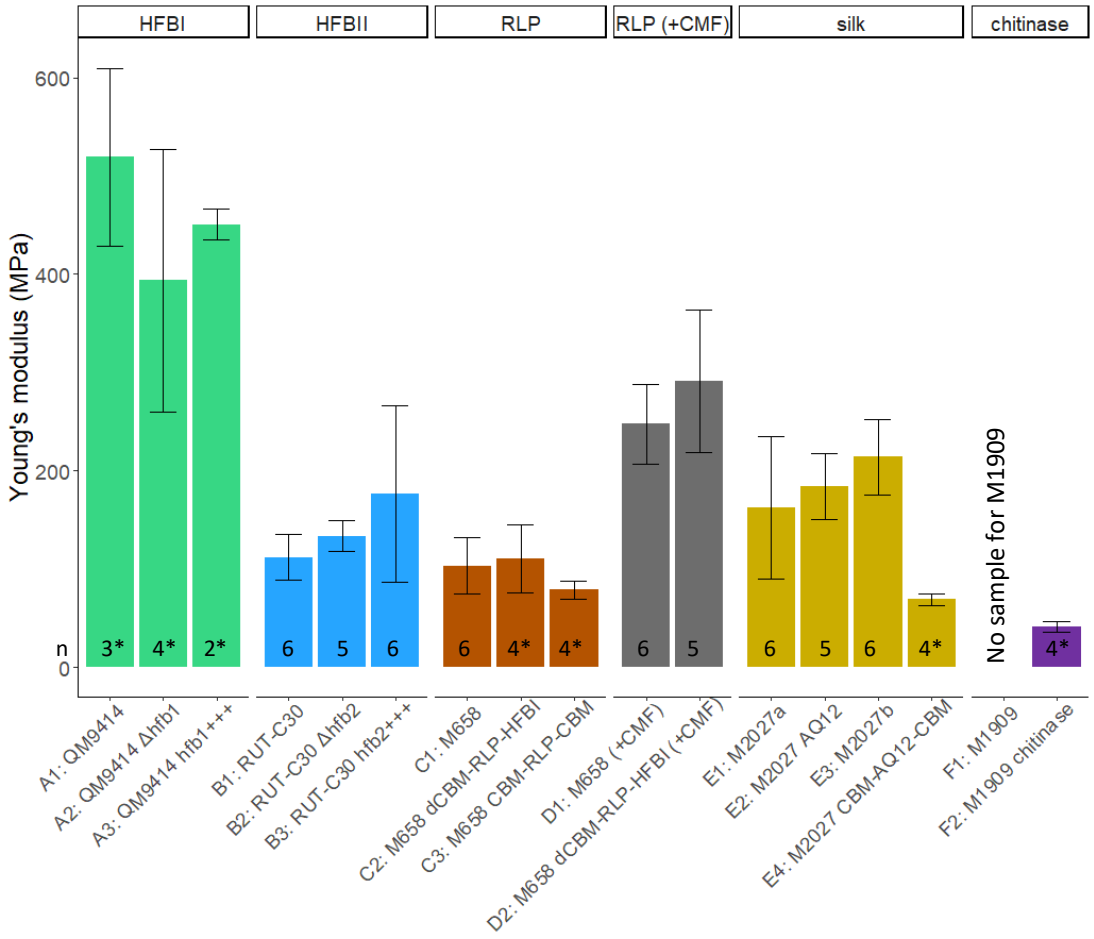


Figure 7. Young's modulus for the mycelium films of the studied *T. reesei* strains. M2027a was the reference for the mycelium film produced by M2027 AQ12, and M2027b was the reference for the mycelium film produced by M2027 CBM-AQ12-CBM. The mycelium composite film samples have "(+CMF)" in their name. The error bars were calculated as the standard deviation within each sample. The number of measure points (n) for each sample film has been marked at the bottom of the graph ranging from two to six. *Four or less measurement points.

To see the differences within sample groups, the absolute values of UTS, strain at UTS and Young's modulus for each sample were compared to each respective reference strain and these relative values are shown in Figure 8. Overall, five samples had p-values less than the set significance level of 0.05 ($p < \alpha = 0.05$), indicating a significant difference in the samples in certain mechanical properties in respect to their reference. Firstly, UTS for QM9414 *Δhfb1* had decreased by 57 % (± 35 pp), while for Rut-C30 *Δhfb2* UTS had increased by 44 % (± 26 pp) and strain at UTS had increased by 37 % (± 51 pp). Thus, opposite effects for the UTS change in hydrophobin deletion strains were observed and only the *hfb1* deletion strain had an increased strain at UTS. Secondly, UTS had increased by 56 % (± 14 pp) and strain at UTS had increased by 10 % (± 23 pp) for M658 dCBM-RLP-HFBI (+CMF), while no significant

differences were observed within the RLP group to be cast as pure mycelium film. Thirdly, UTS for M2027 AQ12 had increased by 48 % (± 28 pp), while UTS had decreased by 58 % (± 12 pp) and Young's modulus had decreased by 69 % (± 19 pp) for M2027 CBM-AQ12-CBM. Thus, the AQ12 producing strain had an increase in UTS while the silk fusion protein producing strain had a decrease in both UTS and Young's modulus.

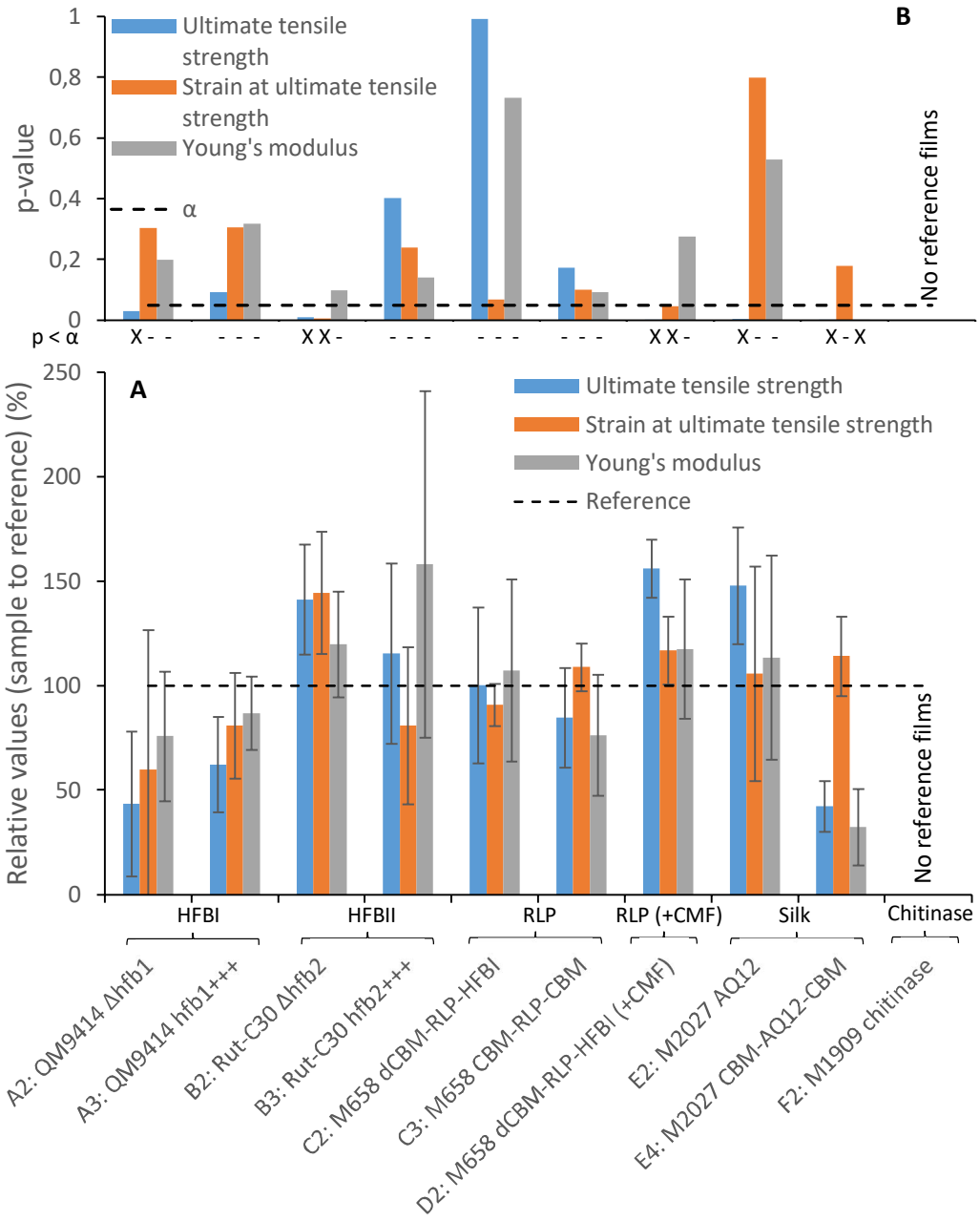


Figure 8. Relative values of UTS, strain at UTS and Young's modulus for each mycelium film produced by the studied *T. reesei* fungal strains in comparison to its respective reference mycelium film (A), and the determined p-value for that comparison (B). The error bar (in A) was calculated as the sum of the standard deviations of the sample and its reference in relation to the mean of the reference. A dashed line (in A) for reference at 100 % indicates the point where the sample and the reference would be equal. The p-value (B) for each sample was determined using the two-sample Student's t-test comparing each sample with its reference assuming unequal variance, normality and independent and individually distributed data. The significance level (α) at 0.05 has been denoted with a dashed line (in B). If p-value is below the alpha level of 0.05 ($p < \alpha$), X has been added below the graph, otherwise a line (-) has been added. There was not a reference sample (M1909) for sample M1909 chitinase and thus relative values nor the p-value could not be determined for them.

The separate repeated cultivations of M2027 for the pure mycelium films and the mycelium-CMF composite films were compared to assess the reliability of the mechanical property determinations with two-sample Student's t-test with unequal variance, and the results for the comparison can be seen in Figure 9. An extremely low p-value of 0.0008 was obtained for the comparison of the UTS values, while strain at UTS and Young's modulus had values above the significance level (α) of 0.05 but still below 0.30. Thus, there was both statistically significant and non-significant but notable evidence against the reliability of the mechanical measurements. However, more repeated samples would be needed to assess the reliability of the analysis method particularly since the differences could have been originated from any point in the whole film production process. These results were still good preliminary indicators.

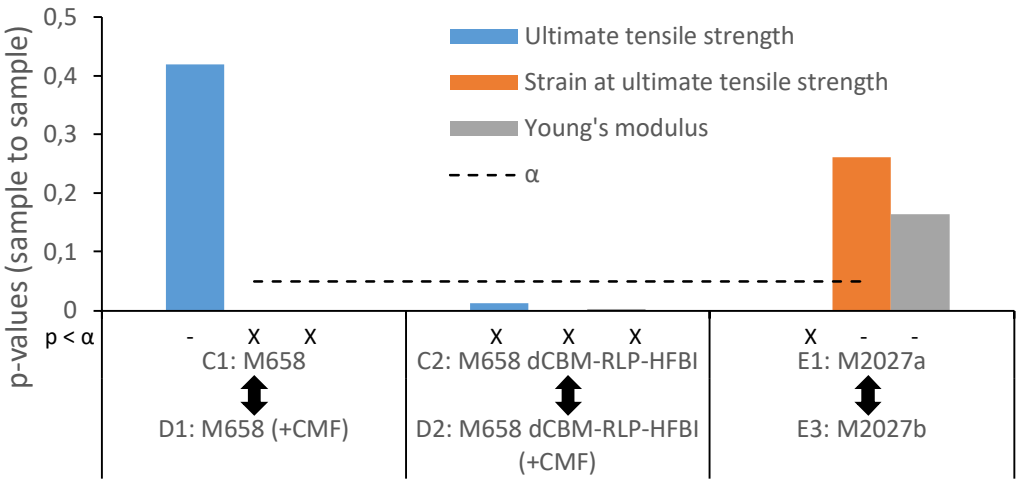


Figure 9. The individual repeated *T. reesei* cultivations and the p-values for the comparisons of the same fungal strains regarding the mechanical properties. The p-value for each sample was determined using two-sample Student's t-test comparing each sample with its respective separately cultivated sample of the same fungal strain. Unequal variance, normality, and independent and individually distributed data was assumed for the test. The significance level (α) at 0.05 was denoted with a dashed line. If a p-value was below the significance level of 0.05 ($p < \alpha$), X was added below the graph, otherwise a line (-) was added.

The separate repeated cultivations of M658 and M658 dCBM-RLP-HFBI were compared to determine the differences caused by the CMF and the results for the comparison are presented in Figure 9. Only the UTS of separate M658 had a p-value (0.4191) above the significance level (α) of 0.05, meaning that there was significant evidence for the difference of mechanical properties of the separately cultivated pure mycelium and mycelium-CMF composite films of the same fungal strains. Thus, CMF significantly decreased strain at UTS and increased Young's modulus for both M658 and M658 dCBM-RLP-HFBI, while CMF only

significantly increased UTS for M658 dCBM-RLP-HFBI. Again, the differences could have been caused at any point in the whole film production process, not just because of the CMF addition, but CMF was the most apparent difference.

6.5 Cell wall polysaccharide content of mycelium

An analytical method to determine the polysaccharide composition of fungal samples was developed in collaboration with other researchers. The samples were divided in four groups for the hydrolysation process of the cell wall polysaccharide content analysis, and a separately cultivated Rut-C30 mycelium biomass was used as a reference in all four separate hydrolysation treatment groups. Only one of the cell wall polysaccharide content results were shown for Rut-C30 to keep the statistics between samples comparable. The first Rut-C30 reference sample for the polysaccharide content analysis was determined to be an outlier within the reference samples and the polysaccharide determinations controlled by it were rejected by the IQR method (data not shown). Thus, cell wall polysaccharide content results were not obtained for strains QM9414, QM9414 Δhfb1, QM9414 hfb1+++ nor Rut-C30 Δhfb2 due to a likely malfunction in sample injection of the HPIC equipment.

The results for the polysaccharide content analysis are shown in Figure 10 as percentage of total cell wall. The mean cell wall total polysaccharide content of all measured samples was 42 % (± 11 pp). On average, glucans constituted for 25 % (± 5 pp), galactomannans for 13 % (± 3 pp), chitin for 4 % (± 4 pp) of the total cell wall dry weight. Surprisingly, chitin was not detected at all in the mycelium cell wall samples for four fungal strains: M658 (+CMF), M2027 CBM-AQ12-CBM, M1909 and M1909 chitinase. Furthermore, chitin content level was overall the lowest of all determined polysaccharides in all samples, and it was apparent that the changes in chitin level had the highest uncertainty of all determined polysaccharides.

The treatment steps for the cell wall polysaccharide content analysis were extensive and presented multiple potential error sources: Mycelium washing, freeze-drying, and grinding were intensively manual and time-consuming treatment steps demanding high attention. Based on the visual inspection by eye, the resulting dry ground fungal cell wall powder was very homogenous right before the hydrolysis steps. However, unlike the pure chitin reference samples, the mycelium cell wall culture samples did not dissolve completely during

hydrolysation (data not shown). This was expected and it was likely, based on the consistency of the results, that the dark brown and black residue particles was mostly cell wall proteins and lipids – and not saccharides. It is possible that some of the cell wall saccharides still got stuck to this residue, but the results indicated that it was at least not that extensive.

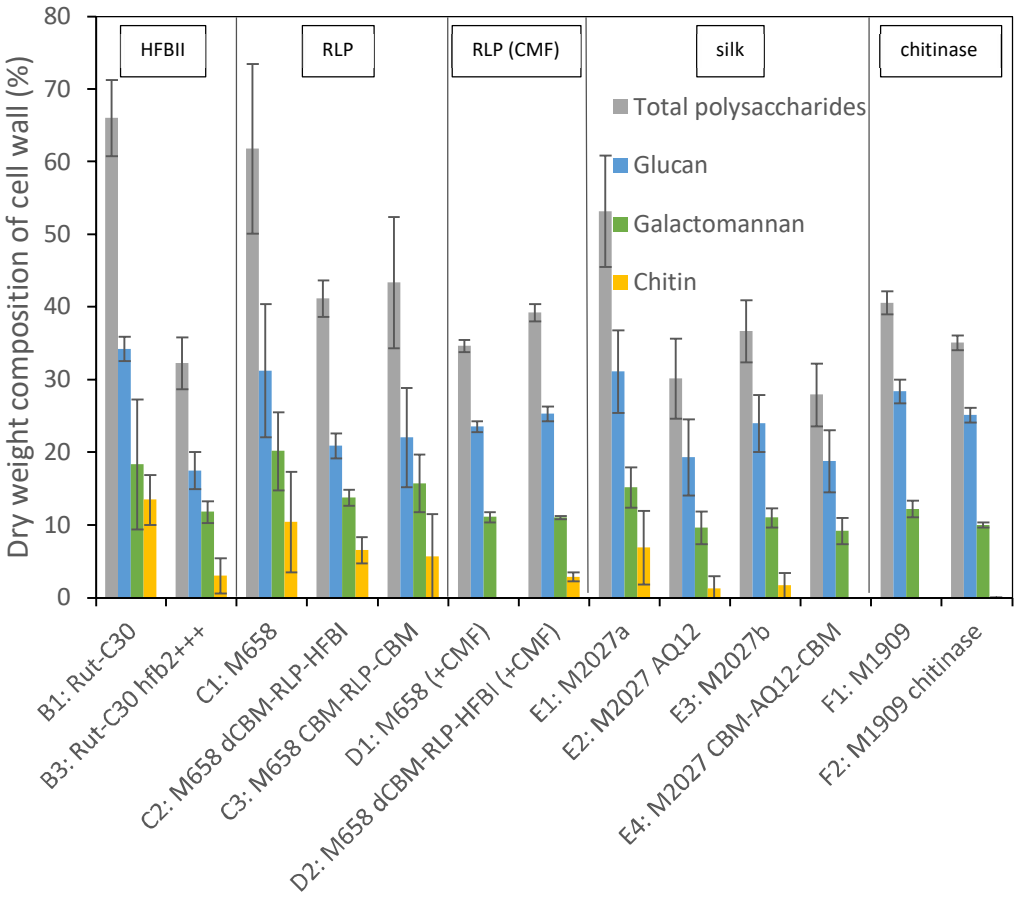


Figure 10. The cell wall polysaccharide dry weight composition of the studied *T. reesei* strains. The analysis method determined polysaccharide amounts per cell wall dry weight as described in the Materials and methods section. The error bars were calculated as the standard deviation within each sample. All the samples had three parallel samples that were treated separately starting with the hydrolysation.

Student's t-test was conducted to determine significant differences and the results both for p-values and relative polysaccharide content values for each sample in comparison to their references are presented in Figure 11. Only two samples had p-values lower than the chosen significance level 0.05 ($p < \alpha = 0.05$) and those results are discussed next. Firstly, for Rut-C30 *hfb2*+++, the total polysaccharide content had decreased by 49 % (± 9 pp), glucan content by 36 % (± 50 pp) and chitin content by 78 % (± 31 pp), meaning that all polysaccharide levels had decreased for the sample in comparison to its reference. Secondly, for M658 dCBM-RLP-

HFBI (+CMF), 3 % (± 1 pp) of chitin was detected in the dry cell wall and the difference two the reference was significant. However, since no chitin was detected in the reference strain sample and the standard deviation was non-existent, the difference was not comparable and the significance could not be properly assessed after all.

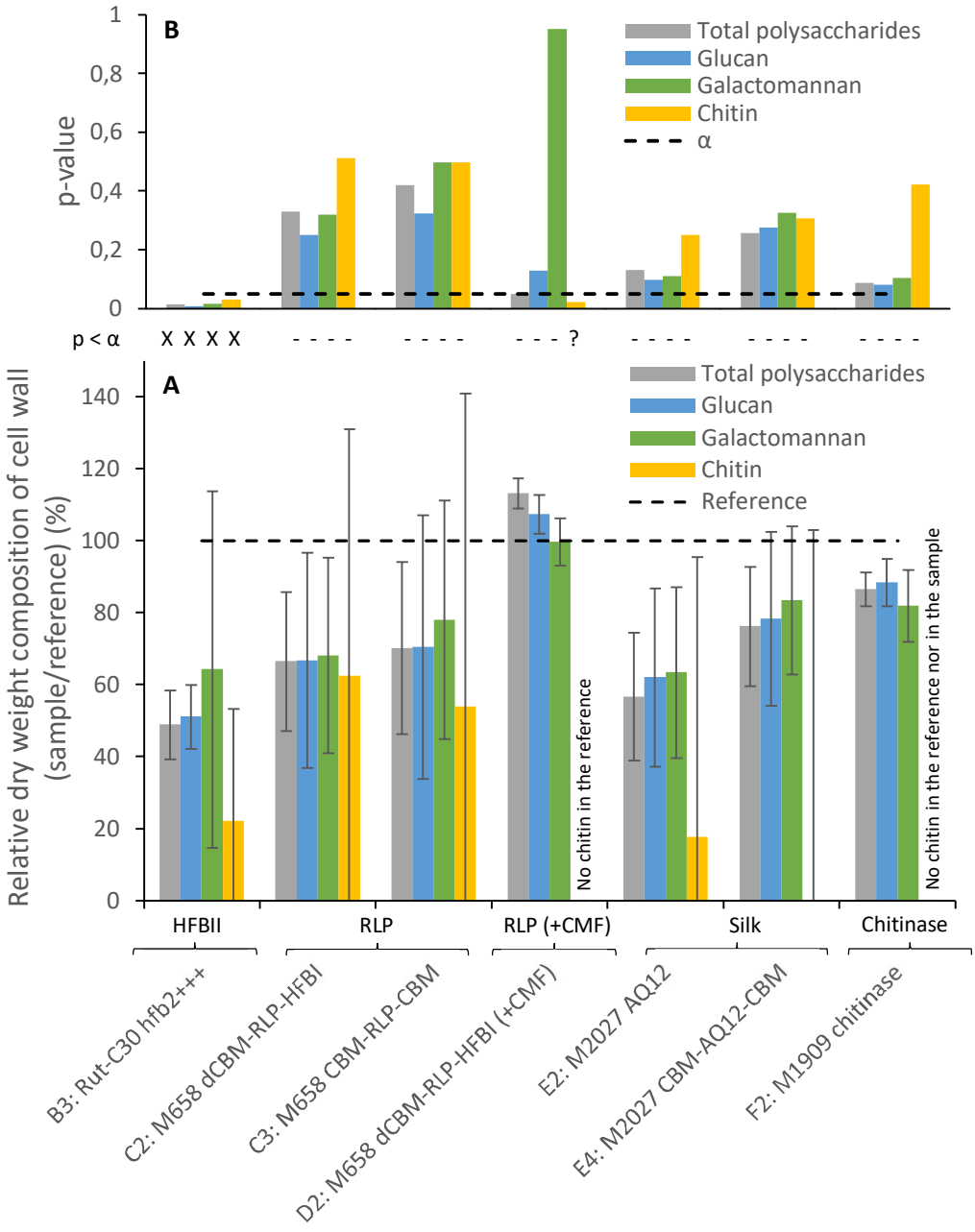


Figure 11. Relative dry weight composition of the cell wall of each studied *T. reesei* sample in comparison to its respective reference strain (A), and the determined p-value for that comparison (B). The error bar (in A) was calculated as the sum of the standard deviations of the sample and its reference in relation to the mean of the reference. A dashed line (in A) for reference at 100 % indicates the point where the sample and the reference would be equal. The p-value (B) for each sample was determined using the two-sample Student's t-test comparing each sample with its reference assuming unequal variance, normality, and independent and individually distributed data. The significance level (α) at 0.05 has been denoted with a dashed line (in B). If p-value was below the alpha level of 0.05 (p < α), X was added below the graph, otherwise a line (-) was added. A question mark (?) was added below the graph if the p-value was obtained by comparing to a zero-level chitin value since it was not a good comparison with a very low or non-existent standard deviation.

The separate repeated cultivations of M658, M658 dCBM-RLP-HFBI and M2027 for the pure mycelium films and the mycelium-CMF composite films were compared to assess the reliability of the cell wall polysaccharide content analysis with two-sample Student's t-test with unequal variance. A p-value of 0.0473 was obtained for the comparison of the separately cultivated M658 dCBM-RLP-HFBI samples regarding the cell wall glucan content, while other values were above the significance level (α) of 0.05, as can be seen in Figure 12. Additionally, the p-values were low and mostly below 0.40, which indicated that there was evidence for the unreliability of the polysaccharide content analysis. However, more data would be needed to evaluate this properly particularly since the differences could have been originated from any point in the whole film production process.

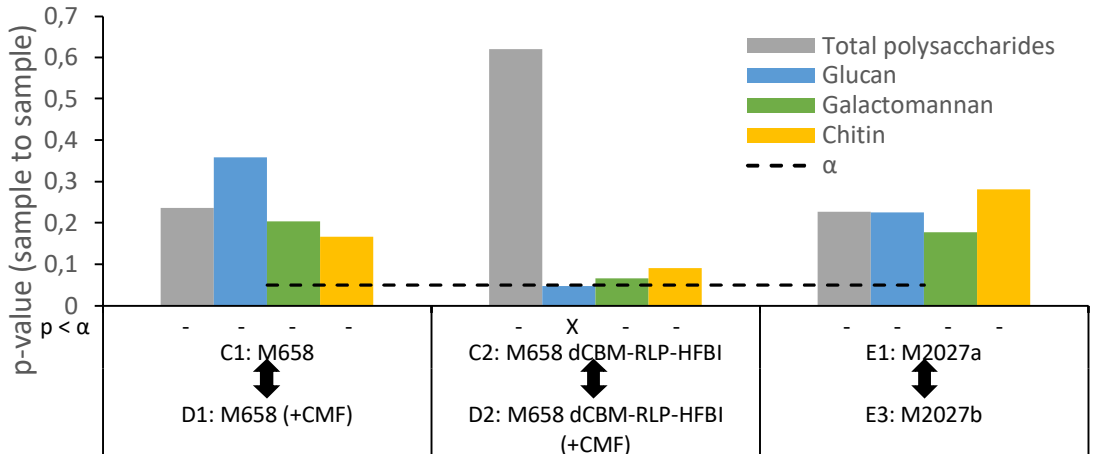


Figure 12. The separate repeated *T. reesei* cultivations and the p-values for the comparisons of the same strains regarding the cell wall polysaccharide contents. The p-value for each sample was determined using two-sample Student's t-test comparing each sample with its respective separately cultivated sample of the same fungal strain. Unequal variance, normality, and independent and individually distributed data was assumed for the test. The significance level (α) at 0.05 was denoted with a dashed line. If a p-value was below the alpha level of 0.05 ($p < \alpha$), X was added below the graph, otherwise a line (-) was added.

The four Rut-C30 control samples were utilised to check how reliable the results for the cell wall polysaccharide content analysis were since they were from the same cultivation, and they went through the same treatment right until the hydrolysis process for the cell wall polysaccharide content analysis. The results for Student's t-test are in Figure 13. The low p-values for the first Rut-C30 control in comparison to all the other Rut-C30 samples demonstrated once again that the first control sample for Rut-C30 was poorly comparable to other control samples, and that its rejection was justified by the IQR method. The p-values between the second, the third and the fourth Rut-C30 samples were all above the 0.05

significance level (which was not a huge requirement) but still generally around 0.5, which meant that while there was uncertainty in the results for the cell wall polysaccharide content analysis, at least the differences were not significant.

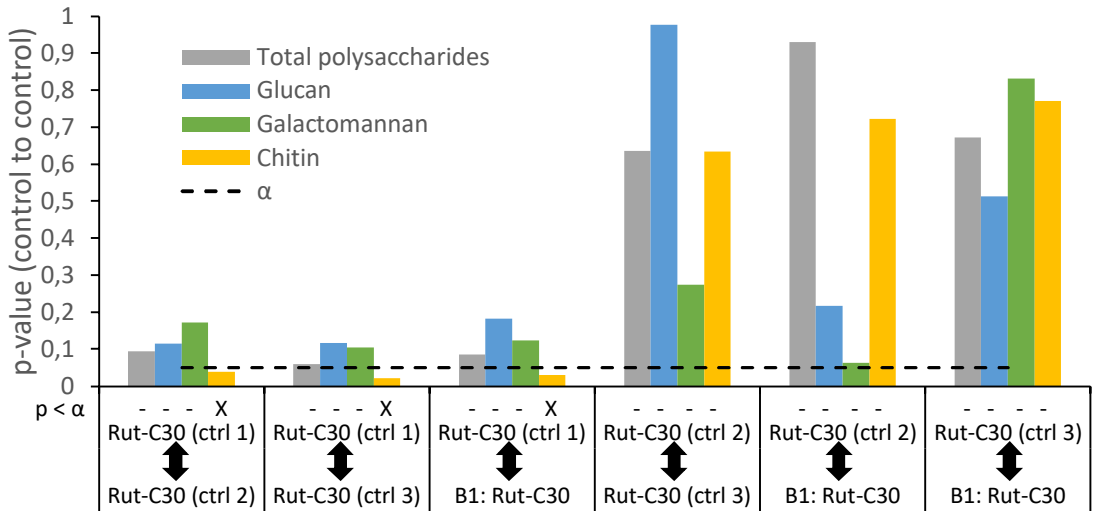


Figure 13. The p-values for comparisons of all four Rut-C30 control samples that were hydrolysed separately for the cell wall polysaccharide content analysis. The control samples were cultivated and treated simultaneously until the hydrolysation process, so that they better control the actual test and the hydrolysation process. The p-value for each control sample was determined using two-sample Student's t-test comparing each control sample with each other. Unequal variance, normality, and independent and individually distributed data was assumed for the test. The significance level (α) at 0.05 was denoted with a dashed line. If a p-value was below the alpha level of 0.05 ($p < \alpha$), X was added below the graph, otherwise a line (-) was added. "B1: Rut-C30" was the fourth control in the analysis. The first control was rejected since it deviated too much from the other controls, as can be seen in the figure as low p-values.

7 Discussion

Sustainable bio-based material solutions are essential in tackling the current and future environmental challenges. The increase interest in bio-based materials has encouraged a strong increase in commercial and research activities related to mycelium materials in the last few decades. Mycelium is inherently biodegradable and is in some cases antimicrobial and non-cytotoxic. Mycelium exhibits many desirable properties, such as good elasticity and strength, fitting for leather-like materials. Furthermore, mycelium with various material properties can be achieved by different fungal species, cultivation conditions, additives, processing methods and post-processing treatments and genetic engineering. This study focused on non-woven mycelium fabric materials in establishing a framework for understanding the effect of both native and recombinant genes on the material properties.

Mycelium is in principle more controllable and adjustable in its material properties than many current synthetic or bio-based material alternatives. This is because mycelium material properties are defined and confined by the genetics driving the regulation and the biochemistry of fungal cells and ultimately the microstructure of mycelium. Moreover, mycelium is not something fungal cell secrete outside the cells but the vegetative and a living part of the organism with apparent intention in its growth patterns and direction in three-dimensional space.

Genes dictate what is produced and when, and therefore they define the cell shape, size, and composition. The regulation of gene activity and metabolic pathways is complex, and relatively poorly known in filamentous fungi. Different fungal species have demonstrated a range of mycelium material properties. There are some studies hinting that even singular gene differences can affect mycelial growth patterns and even mycelium material properties, most accurately density, but this has not been confirmed efficiently. Thus, this thesis further investigated the relationship between genetic engineering and material properties of mycelium.

7.1 Process development and limitations

An important aspect of this work was to further develop the production and the analysis methods for mycelium. Thus, an emphasis was in a consistent processing across samples and on understanding the measurement error. Additionally, the strains in this study were only a small selection of strains that could offer valid reference points in genetic differences. Possible strain-based biases in the results where alleviated and the genetic play field (and the number of differently expressed genes) was expanded by studying several engineered strains and their diverse parent strains as references. The film production was first optimized for Rut-C30, but the process may not have been optimal for the other strains.

The mycelium yield in a practical cultivation setup was challengingly low for all fungal strains except for Rut-C30-derived strains. The lower-than-expected mycelium yields were most likely caused by the cultivation media not being optimal and the utilization of lactose as the carbon source (except for the strains in the HFBI sample group, which were grown with glucose). Thus, fewer replicate samples and measurements could be done, the film size had to be reduced for the HFBI sample group, and for M1909 preparation of a film was not possible. Another possible source of error was the homogenisation of casting suspension and visually different viscosity of the mycelium suspensions, which could have caused some heterogeneity in the produced films, which in turn could have affected the repeatability and reliability in the determination of physical and tensile properties.

7.2 Analyses and limitations

The main limitation for the generalizability of the results of this work was the small sample size (between 1 and 6 measurement points) for all analyses. There were other recognized limitations in the analysis methods of this work: microscopy and the protein expression analyses were qualitative and relative in their nature, so absolute values were not obtained. Or, in the western blot analysis, either a technical error or a proteolytic degradation of the target protein could have hindered the antibody binding and detection for the RLP (+CMF) and silk sample groups.

Density was the key material property and UTS, strain at UTS and Young's modulus were the key mechanical properties determined in this work. The reliability, the validity and ultimately

the possible significance of the results for the physical and mechanical property analyses, and the polysaccharide content analysis were assessed with Student's t-test. The IQR method was used to detect outliers for physical and mechanical property and polysaccharide content determinations. The number of accepted measurement points could be as low as two, since inconsistencies in the mycelium films caused many rejections of parallel film slice samples with the IQR method. A higher IQR rejection rate could have indicated a higher heterogeneity in those samples. The reliability of density values was difficult to assess, since only one measurement point was available.

7.3 Effect of HFBI expression on mycelium properties

The effects of the *hfb1* gene on mycelium properties were studied by the *hfb1* deletion strain (non-expression) and the *hfb1* multicopy strain (overexpression) by comparing them to the wild-type strain QM9414. There were no significant differences in the microstructures nor the yields of the mycelium between the strains and the reference. The hyphae were long with few branching points.

The expected protein expression patterns for the HFBI were confirmed, and that the produced HFBI was bound to the mycelium. Unfortunately, the cell wall polysaccharide content for the HFBI sample group failed, so the genetic effects on that could not be assessed.

Mycelium films were prepared from all three strain in the HFBI sample group. The film size was 40 % of the film size of the other sample groups while the other casting parameters including the drying time was the same. Indeed, the overall highest IQR measurement point rejection rates (between 33 and 66 %) for physical and tensile measurements were within the HFBI sample group, which indicated that there were problems with the homogeneity of the films. However, the film thickness was not significantly different between strains suggesting that the casting might not have been the cause.

The only significantly different mechanical property within the HFBI sample group was the 57 % reduced ultimate tensile strength value of the *hfb1* deletion fungal strain at 3.3 MPa. Young's modulus values were the highest within the HFBI sample group, which was clearly a result from the lowest strain at UTS values among all the studied fungal strains. However,

the UTS values of the modified HFBI fungal strains stayed at a similar level to others. The reference strain QM9414 had the highest UTS potentially increasing the Young's modulus. The higher Young's modulus suggested stiffer characteristics of mycelium hyphae or their reduced ability to bind sorbitol and water for plasticizing. It would have been interesting to study the water absorption properties and plasticizing effects of sorbitol for these HFBI strains.

No significant correlation could be found with the amount of HFBI production and the mechanical properties of the mycelium films. One possible explanation could be that the three strains differed also in other aspects than just the HFBI expression. This could have been a side effect from the gene integration and transformant selection process during strain engineering.

7.4 Effect of HFBII expression on mycelium properties

Similarly to the HFBI sample group, the hydrophobin expression of the *hfb2* gene was studied with the *hfb2* deletion and overexpression strains that were then compared to their parent strain Rut-C30. Again, there were no significant differences in the mycelium yields nor the microstructures within the HFBII sample group. The HFBII sample group had the highest mycelium yields of all the samples in the study. The mycelium hyphae within the HFBII sample group were long with few branching points, as were the hyphae of the fungal strains within the HFBI sample group.

The relative protein expression levels of HFBII were confirmed to be as expected by western blotting. In these cultivation conditions, HFBII was mostly bound to mycelium. HFBII was only additionally produced to the culture suspension outside the cell in the case of the overproduction.

Unfortunately, the cell wall polysaccharide content analysis failed for the *hfb2* deletion strain. Interestingly, the *hfb2* overexpression strain had significantly different cell wall polysaccharide content results to the reference strain for all the measured polysaccharides (glucan, galactomannan, chitin, and the total polysaccharide content). The glucan content in the *hfb2* overexpression strain was approximately half, the galactomannan content two

thirds and the chitin content one fifth of the reference strain. Overall, the overproduction of HFBII protein significantly reduced the polysaccharide contents of the cell wall. This could have been caused by an overload in the overall protein production capability leading to the lack of resources for the CWI pathway processes and synthesisation of the polysaccharose cell wall components.

Regular mycelium films for physical and mechanical property analyses were cast for all the three fungal strains in the HFBII sample group. The thickness of the *hfb2* overexpression strain was significantly higher than the reference strain, indicating that the casting process was not similar between these strains. This could affect the comparability of the results. The number of the measurement points for both the physical and mechanical analyses were high, suggesting that the films were homogenous. An especially interesting observation was the correlation between the film density and the production level of HFBII: the higher the HFBII production, the lower the density. This finding cohered with the article by Appels et al. (2018). However, there was only one measurement point and no parallel samples, so significance of this finding could not be determined. Furthermore, similar relationship between the density and a hydrophobin production level could not be determined for the HFBI sample group. However, if this finding was indeed true, it could mean that HFBII proteins could affect the aggregation of hyphae in mycelium, or the content of bound water and sorbitol within the mycelium.

The only significantly different mechanical properties within the HFBII sample group were observed for the *hfb2* deletion strain with approximately 40 % increased UTS and strain at UTS in comparison to the reference. It is possible that these tensile properties directly reflected the increased material density.

7.5 Effect of RLP expression on mycelium properties

Two different RLP fusion proteins were investigated for the effect on their expression on the mycelium properties both without and with CMF. The mycelium yield and microstructural properties were similar across the RLP and RLP (+CMF) sample groups. Mycelium was more branched and hyphae shorter in these sample groups than in the Rut-C30-based and QM9414-based strains. This was expected for these highly modified strains.

The protein expressions of the RLP fusion proteins were studied for both the RLP and the RLP (+CMF) group. It was confirmed that dCBM-RLP-HFBI was bound to mycelium while CBM-RLP-CBM was released in the suspension. This was in line with the previous studies and the HFBI results of this experiment. However, a reliable western blotting could be utilized for the detection of the protein only for the samples having CMF added to them. For the cultivations without CMF, uncertain SDS-PAGE gels had to be utilised. The HFBI part in the dCBM-RLP-HFBI fusion protein likely caused the protein to remain on the cell wall due to the hydrophobicity of the HFBI or due to the typical transportation methods for HFBI in fungal cells. Overall, the protein expression of the RLP fusion proteins in the mycelium mass without CMF addition seemed low in comparison to an available unpublished research data (Szilvay, G., personal communication). This might have been caused by the use of lactose as a weakly inducing carbon source. Another possibility could be an incomplete detection of the fusion proteins caused by a proteolytic degradation of the Strep-tag.

Furthermore, it was highly likely that similar level of RLP fusion proteins were produced by the strains in the RLP (+CMF) sample group to the strains in the RLP sample group, although this could not be confirmed by western blotting. Similar mycelium yield levels across both sample groups supported this assumption. Since the dCBM-RLP-HFBI fusion protein was observed to be bound to mycelium and less proteolytically degraded than CBM-RLP-CBM, it was chosen as a candidate for the investigation of the possible effects of the RLP fusion protein integrated within mycelium-CMF composite film.

Significant differences were not observed in the cell wall polysaccharide contents in the RLP sample group. In the RLP (+CMF) sample group the chitin content was significantly smaller than in the reference. However, the uncertainty with the chitin determination in this case was high since no chitin was detected in the cell wall of the reference strain in the RLP (+CMF) sample group.

Two separate cultivations were used to cast two kinds of mycelium films: pure mycelium films for the RLP sample group and mycelium-CMF composite films for the RLP (+CMF) sample group. CMF was utilized in the composite films to see how the CBMs in the fusion proteins could be utilized in the enhancement of material properties of mycelium films. There

were significantly different thickness values in both the RLP and the RLP (+CMF) group suggesting that the casting process was inconsistent, which could have had an influence on the results for the mechanical properties of the films.

The density of the pure mycelium films seemed to decrease with increased RLP fusion protein production, while the reverse was observed for the mycelium-CMF composite films. Furthermore, the densities of the composite films were consistently smaller than densities of the pure mycelium films. The cause for this could be less dense CMF fibres, less packed CMF fibres or less packed mycelium hyphae. However, the significance of the differences in the determined densities could not be properly evaluated due to having no replicate samples.

No significant differences in the mechanical properties were determined within the RLP sample group for pure mycelium films. In the case of the M658 dCBM-RLP-HFBI mycelium-CMF composite film sample in comparison to its reference, the UTS had increased by 56 % and strain at UTS had increased by 17 %. These two observed increases were statistically significant. Thus, the dCBM-RLP-HFBI protein production did only affect the mechanical properties significantly in the presence of CMF. This suggested that the cell wall bound CBM-RLP-HFBI successfully interacted with the CMF and/or mycelium within the composite films via CBMs, thereby increasing the toughness of mycelium. In a previous study, the protein CBM-RLP-CBM was observed to similarly crosslink cellulose nanofibrils in material film samples (Fang et al., 2017).

7.6 Effect of silk protein expression on mycelium properties

One silk protein and one silk fusion protein producing fungal strain were compared to their parent fungal strain M2027 to assess the effects of silk protein production on the mycelium properties. No significant differences were observed for the mycelium yields nor the microstructure within the silk sample group. The mycelium yield was around 3 g/L for all strains in the silk sample group. The microstructure of the mycelia within the silk sample group was like the mycelia within the RLP sample group: hyperbranching and short hyphae with bead-like structures.

The protein expression of the silk proteins remained inconclusive in the experiments. Either the desired proteins were not produced, the yields were too low, proteases had degraded the Strep-tag or the western blotting analysis had failed for technical reasons. Unfortunately, the lack of a positive control prevented from ruling out an experimental error. There were slight indications for the silk production in the SDS-PAGE gels, as stronger bands in the expected molecular weight region were observed in the culture supernatant fraction of the silk producing samples in comparison to their reference strain without silk production capability.

The cell wall polysaccharide content analysis yielded non-significant results for the whole silk sample group. However, there was an indication that the cell wall dry content level for all measured polysaccharides had decreased because of the production of the silk proteins, when compared to the parent strain. The same effect was observed for the other genetically engineered strains.

Two separate reference mycelium film samples and one mycelium film sample for each silk strain was prepared. Significant differences were determined in the film thickness values within the silk sample group, which suggested inconsistency in the casting process with possible impact on mechanical properties of the mycelium films. There was a slight indication that the production of the silk proteins decreased density.

There were one significant difference and two significant differences in the mechanical properties of the mycelium films within the silk sample group. The production of AQ12 had significantly increased UTS of the mycelium film by 48 % in comparison to the reference, while the production of CBM-AQ12-CBM significantly decreased UTS and Young's modulus of the film by 58 % and 68 %, respectively. Thus, it seemed like the fusion protein CBM-AQ12-CBM had a decreasing effect while the silk protein AQ12 had an increasing effect on the mechanical properties of the mycelium films. The reason for this might have been that the CBMs in the fusion proteins hindered the packing of the mycelium.

Since the presence of the silk protein could not be confirmed and it seemed more likely that the silk proteins were associated only with the supernatant in the SDS-PAGE gels, it remained unclear what caused the differences in the mechanical properties. A possible cause could

have been the genetic engineering of the strains and its possible side effects on the cell wall composition, although these were not apparent at the microstructural level nor in the mycelium yields, nor did the polysaccharide content analysis strongly suggest this.

7.7 Effect of chitinase expression on mycelium properties

The chitinase overproducing strain was compared to its parent strain to assess the effect of the production of the native chitinase on the mycelium properties. The mycelium yield of the M1909 chitinase strain was over five times the mycelium yield of its reference strain M1909 that had an extremely low mycelium yield of 0.4 g/L. This was confirmed with repeated cultivations. Thus, a mycelium film could not be cast for the chitinase reference strain, and the physical and mechanical properties of the mycelium could not be determined in respect to the chitinase production level and the genetic engineering.

The microstructural differences between these two strains within the chitinase sample group were minimal. They were similar to the microstructure of mycelium within the RLP and the silk sample groups with seemingly a bit shorter hypha length. The microstructure was harder to evaluate due to the low mycelium yields.

The detection of the chitinase protein was not possible by western blotting as no antibody was available, so the SDS-PAGE was used instead. Native cellulases secreted in lactose conditions were not detected in the SDS-PAGE gel most likely due to the low mycelium yield of M1909 parent strain. The difference in mycelium yields made it harder to compare the gels of the parent and the reference. Based on the previous unpublished research data, it seemed likely that M1909 chitinase strain indeed produced more chitinase than the M1909 reference strain.

The cell wall polysaccharide content levels of M1909 chitinase strain did not differ significantly from the M1909 reference strain. However, the total polysaccharide content, glucan content and galactomannan content seemed to be reduced for the M1909 chitinase strain, while the chitin content remained at the zero level. No strong evidence was found for the reduction. It could be possible that the overproduced chitinase caused a change in the

chitin content of the cell wall, but the original chitin content was already very close to the lower detection limit of the analysis method.

The mycelium film density of M1909 chitinase was 310 kg/dm^3 and at the similar level to the other strains. The film thickness of M1909 chitinase was 277 µm, which was also similar to the other strains. A comparison was not possible due to the lack of the reference film sample.

UTS and Young's modulus seemed to be the lowest among all samples for M1909 chitinase fungal strain. The general level of strain at UTS for the M1909 chitinase sample was close to that of the RLP sample group. It would have been valuable to be able to produce a proper reference film for M1909 chitinase film sample in order to determine the effect of the chitinase over-production on the mycelium properties.

7.8 Other effects on mycelium properties

In this work, the average cell wall glucan content of 25 % and the average cell wall chitin content of 4 % were determined. In comparison, (Kappel and Gruber, 2020) reported the cell wall glucan content of around one third for *T. reesei* in their study, while the cell wall chitin content was at the same level. The average cell wall galactomannan content of 13 % determined in this work was around half of the level reported by Gastebois et al. (2009) for *A. fumigatus* (a reference for *Trichoderma* was not available). The total polysaccharide cell wall dry content of this work was on average less than half of the total cell wall dry content. This indicates that the dry cell wall consisted mostly of proteins, if other impurities were indeed not present and the amount of lipid was low, as presumed. This result was approximately 20 pp less than the 30 % proteinaceous dry cell wall content reported by Kappel and Gruber (2020) for *Trichoderma* spp. The reason for the observed differences could be that either the strains in this experiment were notably different in their cell wall content in relation to the strains utilized in the literature, or that there was uncertainty in the results.

Sorbitol was the only additive studied in this thesis, while post-treatment methods were not utilized on the mycelium films. It would have been interesting to study the possible effects of alternative additives (crosslinkers, humectants, plasticizers) and post-treatment methods

(tanning, coating) on mycelium, since they have a huge potential in increasing the mechanical properties of mycelium films (Jones et al., 2021), but that was outside the scope of this work.

The cell wall polysaccharide content results suggested that the genetic engineering of the studied fungal strains had reduced the content of all polysaccharides in the cell wall, and significant results for this reduction were found for the overproduction of the HFBII protein. Additionally, the density seemed to be reduced for the genetically engineered strains. This phenomenon is not well understood in *T. reesei,* but some literature indicates a link between protein secretion, glycosylation, and cell wall composition in fungi (Tang et al., 2016; Perlińska-Lenart et al., 2006). However, the comparisons for separately cultivated M2027, M658 and M658 dCBM-RLP-HFBI samples suggested that the reliability of the cell wall polysaccharide content needs to be further evaluated. Density values had only one measurement point and thus their reliability could not be properly assessed.

Overall, the results indicated that there were either no significant differences in the mechanical properties between the genetically engineered fungal strains and their respective references, or that the mechanical properties were lower for the genetically engineered strains. In previous studies, it has been shown that the material tensile properties can be significantly increased by the addition of cellulosic fibres (Elsacker et al., 2021; Fang et al., 2017). While the purpose of this thesis was to investigate the pure mycelium properties, the CMF was used as an additive to study the effect of the multifunctional dCBM-RLP-HFBI protein as an interfacing protein between cellulose and the mycelium.

The mechanical values determined for the films in this work were low overall: UTS was between 1.4 and 7.6 MPa, Strain at UTS between 2 and 16 %. Some literature values for mycelium film tensile properties range between 0.5 and 1.5 MPa UTS and 20 and 100 % strain (Bustillos et al., 2020; Haneef et al., 2017). The mycelium films prepared in this work displayed higher UTS but lower strain values than in literature. Interestingly, wet-spun mycelium filaments could have Young's modulus of up to 4.97 GPa and UTS of up to 69.5 MPa (Svensson et al., 2021), as was noted earlier in this work. This did not seem attainable with the straightforward casting method utilized for mycelium films in this work. However, it demonstrated that controlling the orientation of mycelium filaments would be a great way of improving the mechanical properties of mycelium even further.

8 Conclusions and outlook

While the effects of single native genes to mycelium material properties have been studied before (Appels et al., 2018), this was the first time the effects of introducing recombinant proteins in fungal genome on material properties of mycelium were studied, to the author's knowledge. During the progress of this thesis, optimal process methods, parameters and analyses were further contemplated to allow more defined production of mycelium films henceforth. This work helped in identification of possible bottlenecks and challenge points in the production of mycelium films. A method for cell wall polysaccharide content was developed and promising results were obtained, while other methods were further tested.

Additional repeated sample cultivations and more parallel samples could have helped to determine various effects on mycelium more reliably and soundly, but that was out of the scope of this study. The efforts were instead focused on screening of *T. reesei* strains with the various analyses and their development for the future research work.

None of the results were straightforward to interpret and many things needs to be solved in the future to reliably determine the true meaning of these results. The most notable findings were not significant but suggestive in their nature, such as that the genetic engineering seemed to decrease the polysaccharide contents and that the HFBII level seemed to correlate with density. An intriguing finding in the mechanical properties was that CBM-RLP-HFBI seemed to successfully interact with the CMF within the composite films via CBMs increasing the toughness and the elasticity of the mycelium film. It was also demonstrated that wider genetic differences brought apparent microstructural differences, which should at some point yield differences in mechanical properties.

Multiple significant findings were made in the relationship of mycelium material properties in relation to single genetic differences, which typically opened more questions and opportunities for further research work. The most valuable output of this work was the refining of the processing and analysis methods of mycelium films to allow more streamlined research on mycelium materials in the future. The world acutely needs more sustainable material solutions. A more comprehensive understanding and harnessing of the potential of mycelium materials in our everyday life would certainly assist in remedying that need.

9 References

ADRIANO DI MARTI (2022a), *ADRIANO DI MARTI*, Available at: https://desserto.com.mx/adriano-di-marti-1 (Accessed: 18.9.2022).

ADRIANO DI MARTI (2022b), *DESERTTEX - Desserto, Deserttex*, Available at: https://deserttex.com/ (Accessed: 18.9.2022).

Ahokas, P., Penttilä, M., Boer, H., Suhonen, A., Arias Barrantes, M., Laine, C. and Szilvay, G. (2020), Methods Of Making Non-Woven Materials From Mycelium.

Ananas Anam Ltd. (2022), *Piñatex - Piñatex*, Available at: https://www.ananas-anam.com/ (Accessed: 18.9.2022).

Appels, F. V. W., van den Brandhof, J. G., Dijksterhuis, J., de Kort, G. W. and Wösten, H. A. B. (2020), Fungal mycelium classified in different material families based on glycerol treatment, *Communications Biology* **3**(1) 1–5.

Appels, F. V. W., Dijksterhuis, J., Lukasiewicz, C. E., Jansen, K. M. B., Wösten, H. A. B. and Krijgsheld, P. (2018), Hydrophobin gene deletion and environmental growth conditions impact mechanical properties of mycelium by affecting the density of the material, *Scientific Reports* **8** 1–7.

Askolin, S., Nakari-Setälä, T. and Tenkanen, M. (2001), Overproduction, purification, and characterization of the Trichoderma reesei hydrophobin HFBI, *Applied Microbiology and Biotechnology* **57**(1–2) 124–130.

Askolin, S., Penttilä, M., Wösten, H. A. B. and Nakari-Setälä, T. (2005), The Trichoderma reesei hydrophobin genes hfb1 and hfb2 have diverse functions in fungal development, *FEMS Microbiology Letters* **253**(2) 281–288.

Atanasova, L., Gruber, S., Lichius, A., Radebner, T., Abendstein, L., Münsterkötter, M., Stralis-Pavese, N., Łabaj, P. P., Kreil, D. P. and Zeilinger, S. (2018), The Gpr1-regulated Sur7 family protein Sfp2 is required for hyphal growth and cell wall stability in the mycoparasite Trichoderma atroviride, *Scientific Reports* **8**(1) 1–15.

Aufauvre-Brown, A., Mellado, E., Gow, N. A. R. and Holden, D. W. (1997), Aspergillus fumigatus chsE: A gene related to CHS3 of Saccharomyces cerevisiae and important for hyphal growth and conidiophore development but not pathogenicity, *Fungal Genetics and Biology* **21**(1) 141–152.

Avenas, P. (2013), Etymology of main polysaccharide names, *The European Polysaccharide Network of Excellence (EPNOE)* 13–21, Wien, Springer-Verlag.

Bae, B., Kim, M., Kim, S. and Ro, H.-S. (2021), Growth Characteristics of Polyporales Mushrooms for the Mycelial Mat Formation, *Mycobiology,* **49**(3) 280–284.

Bailey, M., Askolin, S., Hörhammer, N., Tenkanen, M., Linder, M., Penttilä, M. and Nakari-Setälä, T. (2002), Process technological effects of deletion and amplification of hydrophobins I and II in transformants of Trichoderma reesei, *Applied Microbiology and Biotechnology* **58**(6) 721–727.

Batys, P., Fedorov, D., Mohammadi, P., Lemetti, L., Linder, M. B. and Sammalkorpi, M. (2021), Self-Assembly of Silk-like Protein into Nanoscale Bicontinuous Networks under Phase-Separation Conditions, *Biomacromolecules* **22**(2) 690–700.

Blanchette, R. A., Haynes, D. T., Held, B. W., Niemann, J. and Wales, N. (2021), Fungal mycelial mats used as textile by indigenous people of North America, *Mycologia* **113**(2) 261–267.

Bolt Threads (2022), *Mylo™ Unleather - Sustainable Vegan Mycelium Leather*, Available at: https://www.mylo-unleather.com/ (Accessed: 18.9.2022).

Boukid, F., Rosell, C. M., Rosene, S., Bover-Cid, S. and Castellari, M. (2022), Non-animal proteins as cutting-edge ingredients to reformulate animal-free foodstuffs: Present status and future perspectives, *Critical Reviews in Food Science and Nutrition* **62**(23) 6390–6420.

van den Brandhof, J. G. and Wösten, H. A. B. (2022), Risk assessment of fungal materials, *Fungal Biology and Biotechnology* **9**(1).

Brock, T. D., Madigan, M. T., Martinko, J. M. and Parker, J. (2003), *Brock biology of microorganisms*, 10th edition, Upper Saddle River (NJ), Pearson Education, Inc.

Bustillos, J., Loganathan, A., Agrawal, R., Gonzalez, B. A., Perez, M. G., Ramaswamy, S., Boesl, B. and Agarwal, A. (2020), Uncovering the Mechanical, Thermal, and Chemical Characteristics of Biodegradable Mushroom Leather with Intrinsic Antifungal and Antibacterial Properties, *ACS Applied Bio Materials* **3**(5) 3145–3156.

Cerimi, K., Akkaya, K. C., Pohl, C., Schmidt, B. and Neubauer, P. (2019), Fungi as source for new bio-based materials: A patent review, *Fungal Biology and Biotechnology* **6**(1).

China, C. R., Maguta, M. M., Nyandoro, S. S., Hilonga, A., Kanth, S. v. and Njau, K. N. (2020), Alternative tanning technologies and their suitability in curbing environmental pollution from the leather industry: A comprehensive review, *Chemosphere* **254**.

Dekking, F. M., Kraaikamp, C., Lopuhaä, H. P. and Meester, L. E. (2005), Exploratory data analysis: numerical summaries, *A Modern Introduction to Probability and Statistics - Understanding Why and How* 231–238, London, Springer.

Ecovative Design LLC (2022a), *MycoComposite - Ecovative*, Available at: https://www.ecovative.com/pages/mycocomposite (Accessed.:

Ecovative Design LLC (2022b), *foragerbio*, Available at: https://forager.bio/ (Accessed: 18.9.2022).

Elsacker, E., Vandelook, S., Damsin, B., van Wylick, A., Peeters, E. and de Laet, L. (2021), Mechanical characteristics of bacterial cellulose-reinforced mycelium composite materials, *Fungal Biology and Biotechnology* **8**(1).

Fakirov, S. (2015), *Composite materials - is the use of proper definitions important?*, *Materials Today* **18**(10), Elsevier B.V.

Fang, W., Paananen, A., Vitikainen, M., Koskela, S., Westerholm-Parvinen, A., Joensuu, J. J., Landowski, C. P., Penttilä, M., Linder, M. B. and Laaksonen, P. (2017), Elastic and pH-Responsive Hybrid Interfaces Created with Engineered Resilin and Nanocellulose, *Biomacromolecules* **18**(6) 1866–1873.

Fazli Wan Nawawi, W. M., Lee, K. Y., Kontturi, E., Murphy, R. J. and Bismarck, A. (2019), Chitin Nanopaper from Mushroom Extract: Natural Composite of Nanofibers and Glucan from a Single Biobased Source, *ACS Sustainable Chemistry and Engineering* **7**(7) 6492–6496.

Feofilova, E. P. (2010), The fungal cell wall: Modern concepts of its composition and biological function, *Microbiology* **79**(6) 711–720.

Fiscatech (2022), *Sustainitaly - Fiscatech*, Available at: https://www.fiscatech.com/sustainitaly/?lang=en (Accessed: 18.9.2022).

Fontaine, T. and Latgé, J.-P. (2020), Galactomannan Produced by Aspergillus fumigatus: An Update on the Structure, Biosynthesis and Biological Functions of an Emblematic Fungal Biomarker, *Journal of Fungi* **6**(4) 1–17.

François, J. M. (2007), A simple method for quantitative determination of polysaccharides in fungal cell walls, *Nature Protocols* **1**(6) 2995–3000.

Fratzl, P. (2007), Biomimetic materials research: What can we really learn from nature's structural materials?, *Journal of the Royal Society Interface* **4**(15) 637–642.

Free, S. J. (2013), Fungal Cell Wall Organization and Biosynthesis, *Advances in Genetics* **81** 33–82.

Fruitleather Rotterdam (2022), *Home - Fruitleather Rotterdam*, Available at: https://fruitleather.nl/ (Accessed: 18.9.2022).

Fuchs, B. B. and Mylonakis, E. (2009), Our paths might cross: The role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways, *Eukaryotic Cell* **8**(11) 1616–1625.

Gamblin, D. P., Scanlan, E. M. and Davis, B. G. (2009), Glycoprotein Synthesis: An Update, *Chemical Reviews* **109**(1) 131–163.

Gandia, A., van den Brandhof, J. G., Appels, F. V. W. and Jones, M. P. (2021), Flexible Fungal Materials: Shaping the Future, *Trends in Biotechnology* **39**(12).

García, C. and Prieto, M. A. (2019), Bacterial cellulose as a potential bioleather substitute for the footwear industry, *Microbial Biotechnology* **12**(4) 582–585.

Gastebois, A., Clavaud, C., Aimanianda, V. and Latgé, J. P. (2009), Aspergillus fumigatus: cell wall polysaccharides, their biosynthesis and organization, *Future microbiology* **4**(5) 583–595.

González, N., Marquès, M., Nadal, M. and Domingo, J. L. (2020), Meat consumption: Which are the current global risks? A review of recent (2010–2020) evidences, *Food Research International* **137**.

Gow, N. A. R., Latge, J.-P. and Munro, C. A. (2017), The Fungal Cell Wall: Structure, Biosynthesis, and Function, *Microbiology Spectrum* **5**(3) 1–25.

Grado Zero Espace Research Lab. (2022), *Grado Zero Espace - New Technologies Research and Development*, Available at: https://www.gzespace.com/ (Accessed: 18.9.2022).

Griffo, A., Hahl, H., Grandthyll, S., Muller, F., Paananen, A., Ilmen, M., Szilvay, G. R., Landowski, C. P., Penttila, M., Jacobs, K. and Laaksonen, P. (2017), Single-molecule force spectroscopy study on modular resilin fusion protein, *ACS Omega* **2**(10) 6906–6915.

Haneef, M., Ceseracciu, L., Canale, C., Bayer, I. S., Heredia-Guerrero, J. A. and Athanassiou, A. (2017), Advanced Materials from Fungal Mycelium: Fabrication and Tuning of Physical Properties, *Scientific Reports* **7**(1) 1–11.

Hildebrandt, J., Thrän, D. and Bezama, A. (2021), The circularity of potential bio-textile production routes: Comparing life cycle impacts of bio-based materials used within the manufacturing of selected leather substitutes, *Journal of Cleaner Production* **287**.

International Organization for Standardization (2020), Leather - Physical and mechanical tests - Determination of tensile strength and percentage elongation (ISO 3376:2020).

Jones, M., Bhat, T., Huynh, T., Kandare, E., Yuen, R., Wang, C. H. and John, S. (2018), Waste-derived low-cost mycelium composite construction materials with improved fire safety, *Fire and Materials* **42**(7) 816–825.

Jones, M., Gandia, A., John, S. and Bismarck, A. (2021), Leather-like material biofabrication using fungi, *Nature Sustainability* **4**(1).

Jones, M. P., Lawrie, A. C., Huynh, T. T., Morrison, P. D., Mautner, A., Bismarck, A. and John, S. (2019), Agricultural by-product suitability for the production of chitinous composites and nanofibers utilising Trametes versicolor and Polyporus brumalis mycelial growth, *Process Biochemistry* **80** 95–102.

Jones, M. P., Wang, C.-H., John, Sabu, Bhat, T., Jones, M., Kandare, E., Yuen, R., Wang, C. H. and John, S (2018), *Biomass and waste-derived sustainable mycelium composite construction materials with enhanced fire safety*.

Jones, M., Weiland, K., Kujundzic, M., Theiner, J., Kählig, H., Kontturi, E., John, S., Bismarck, A. and Mautner, A. (2019), Waste-Derived Low-Cost Mycelium Nanopapers with Tunable Mechanical and Surface Properties, *Biomacromolecules* **20**(9) 3513–3523.

Joshi, K., Meher, M. K. and Poluri, K. M. (2020), Fabrication and Characterization of Bioblocks from Agricultural Waste Using Fungal Mycelium for Renewable and Sustainable Applications, *ACS Applied Bio Materials* **3**(4) 1884–1892.

Kappel, L. and Gruber, S. (2020), Chitin and chitosan—important structural components in Trichoderma cell wall remodeling, *New and Future Developments in Microbial Biotechnology and Bioengineering* 243–280, Elsevier.

Kappel, L., Münsterkötter, M., Sipos, G., Escobar, C. R. and Gruber, S. (2020), Chitin and chitosan remodeling defines vegetative development and Trichoderma biocontrol, *PLoS pathogens* **16**(2).

Kumar, N. S., Suvarna, R. P., Naidu, K. C. B., Banerjee, P., Ratnamala, A. and Manjunatha, H. (2020), A review on biological and biomimetic materials and their applications, *Applied Physics A: Materials Science and Processing* **126**(6) 1–18.

Landowski, C., Huuskonen, A., Saarinen, J., Westerholm-Parvinen, A., Kanerva, A., Natunen, J., Hänninen, A.-L., Salovuori, N., Penttilä, M. and Saloheimo, M. (2013), Protease deficient filamentous fungal cells and methods of use thereof.

Landowski, C. P., Huuskonen, A., Wahl, R., Westerholm-Parvinen, A., Kanerva, A., Hänninen, A. L., Salovuori, N., Penttilä, M., Natunen, J., Ostermeier, C., Helk, B., Saarinen, J. and Saloheimo, M. (2015), Enabling low cost biopharmaceuticals: A systematic approach to

delete proteases from a well-known protein production host trichoderma reesei, *PLoS ONE* **10**(8).

Latgé, J. P. (2007), The cell wall: A carbohydrate armour for the fungal cell, *Molecular Microbiology* **66**(2) 279–290.

Levin, D. E. (2011), Regulation of cell wall biogenesis in Saccharomyces cerevisiae: The cell wall integrity signaling pathway, *Genetics* **189**(4) 1145–1175.

Li, D., Tang, Y., Lin, J. and Cai, W. (2017), Methods for genetic transformation of filamentous fungi, *Microbial Cell Factories* **16**(1).

Linder, M. B., Szilvay, G. R., Nakari-Setälä, T. and Penttilä, M. E. (2005), Hydrophobins: the protein-amphiphiles of filamentous fungi, *FEMS Microbiology Reviews* **29**(5) 877–896.

Lodish, H., Berk, A., Matsudaira, P., Kaiser, C. A., Krieger, M., Scott, M. P., Zipursky, L. and Darnell, J. (2004), *Molecular Cell Biology*, 5th edition, Macmillan.

Malai Eco (2022), *Malai Eco*, Available at: https://malai.eco/ (Accessed: 18.9.2022).

Malavazi, I., Goldmanm, G. H. and Brown, N. A. (2014), The importance of connections between the cell wall integrity pathway and the unfolded protein response in filamentous fungi, *Briefings in Functional Genomics* **13**(6) 456–470.

Mandels, M., Hontz, L. and Brandt, D. (1972), *Disposal of cellulosic waste materials by enzymatic hydrolysis*, Natick.

Mandels, M., Weber, J. and Parizek, R. (1971), Enhanced Cellulase Production by a Mutant of Trichoderma viride, *Applied Microbiology* **21**(1) 152–154.

Mazhandu, Z. S., Muzenda, E., Mamvura, T. A., Belaid, M. and Nhubu, T. (2020), Integrated and consolidated review of plastic waste management and bio-based biodegradable plastics: Challenges and opportunities, *Sustainability (Switzerland)* **12**(20) 1–57.

Meyer, V. (2022), Connecting materials sciences with fungal biology: a sea of possibilities, *Fungal Biology and Biotechnology* **9**(1).

Meyer, V., Basenko, E. Y., Benz, J. P., Braus, G. H., Caddick, M. X., Csukai, M., Vries, R. P. de, Endy, D., Frisvad, J. C., Cimerman, N. G., Haarmann, T., Hadar, Y., Hansen, K., Johnson, R. I. and Keller, N. P. (2020), Growing a circular economy with fungal biotechnology: a white paper, *Fungal Biology and Biotechnology* **7**(5) 1–23.

Modern Meadow (2022), *Modern Meadow*, Available at: https://www.modernmeadow.com/ (Accessed: 18.9.2022).

MoEa (2022), *AppleSkin – MoEa*, Available at: https://moea.io/pages/appleskin (Accessed: 18.9.2022).

mogu (2022a), *Floor Flex - mogu*, Available at: https://mogu.bio/flooring/floor-flex/ (Accessed: 18.9.2022).

mogu (2022b), *Floor Tiles - mogu*, Available at: https://mogu.bio/flooring/floor-tiles/ (Accessed: 18.9.2022).

mogu (2022c), *Acoustic Catalogue - mogu*, Available at: https://mogu.bio/acoustic-catalogue-2022/ (Accessed: 18.9.2022).

Mohammadi, M., Zamani, A. and Karimi, K. (2012), Determination of glucosamine in fungal cell walls by high-performance liquid chromatography (HPLC), *Journal of Agricultural and Food Chemistry* **60**(42) 10511–10515.

Mohammadi, P., Aranko, A. S., Landowski, C. P., Ikkala, O., Jaudzems, K., Wagermaier, W. and Linder, M. B. (2019), Biomimetic composites with enhanced toughening using silk-inspired triblock proteins and aligned nanocellulose reinforcements, *Science advances* **5**(9) 1–11.

Mohammadi, P., Aranko, A. S., Lemetti, L., Cenev, Z., Zhou, Q., Virtanen, S., Landowski, C. P., Penttilä, M., Fischer, W. J., Wagermaier, W. and Linder, M. B. (2018), Phase transitions as intermediate steps in the formation of molecularly engineered protein fibers, *Communications Biology* **1**(1) 1–12.

Mohammadi, P., Beaune, G., Stokke, B. T., Timonen, J. V. I. and Linder, M. B. (2018), Self-Coacervation of a Silk-Like Protein and Its Use As an Adhesive for Cellulosic Materials, *ACS Macro Letters* **7**(9) 1120–1125.

Montenecourt, B. S. and Eveleigh, D. E. (1979), Selective Screening Methods for the Isolation of High Yielding Cellulase Mutants of Trichoderma reesei, *Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis*, Advances in Chemistry **181** 289–301, AMERICAN CHEMICAL SOCIETY.

Moore, D. and Novak, L. F. (2002), *Essential Fungal Genetics*, New York, Springer.

Müller, C., Klemm, S. and Fleck, C. (2021), Bracket fungi, natural lightweight construction materials: hierarchical microstructure and compressive behavior of Fomes fomentarius fruit bodies, *Applied Physics A: Materials Science and Processing* **127**(3) 1–11.

Mycotech Lab (2022), *Mycotech Lab - Mylea*, Available at: https://mycl.bio/mylea (Accessed: 18.9.2022).

MycoWorks (2022), *Home - MycoWorks*, Available at: https://www.mycoworks.com/ (Accessed: 18.9.2022).

Nakari-Setälä, T., Aro, N., Ilmén, M., Muñoz, G., Kalkkinen, N. and Penttilä, M. (1996), Genetic and biochemical characterization of the Trichoderma reesei hydrophobin HFBI, *European Journal of Biochemistry* **235**(1–2) 248–255.

Nakari-Setälä, T., Aro, N., Ilmén, M., Muñoz, G., Kalkkinen, N. and Penttilä, M. (1997), Differential expression of the vegetative and spore-bound hydrophobins of Trichoderma reesei - Cloning and characterization of the hgb2 gene, *European Journal of Biochemistry* **248**(2) 415–423.

Nawawi, W. M. F. W., Jones, M. P., Kontturi, E., Mautner, A. and Bismarck, A. (2020), Plastic to elastic: Fungi-derived composite nanopapers with tunable tensile properties, *Composites Science and Technology* **198** 1–8.

Niaounakis, M. (2015), Introduction, *Biopolymers: Processing and Products* 1–77, Elsevier.

Patil, R. S., Ghormade, V. and Deshpande, M. v (2000), Chitinolytic enzymes: an exploration, *Enzyme and Microbial Technology* **26**(7) 473–483.

Peintner, U., Pöder, R. and Pümpel, T. (1998), The iceman's fungi, *Mycological Research* **102**(10) 1153–1162.

Pelletier, M. G., Holt, G. A., Wanjura, J. D., Greetham, L., McIntyre, G., Bayer, E. and Kaplan-Bie, J. (2019), Acoustic evaluation of mycological biopolymer, an all-natural closed cell foam alternative, *Industrial Crops and Products* **139**.

Perlińska-Lenart, U., Orłowski, J., Laudy, A. E., Zdebska, E., Palamarczyk, G. and Kruszewska, J. S. (2006), Glycoprotein hypersecretion alters the cell wall in Trichoderma reesei strains expressing the Saccharomyces cerevisiae dolichylphosphate mannose synthase gene, *Applied and Environmental Microbiology* **72**(12) 7778–7784.

Peterson, R. and Nevalainen, H. (2012), Trichoderma reesei RUT-C30 - Thirty years of strain improvement, *Microbiology* **158**(1) 58–68.

Prosapio, V. and Lopez-Quiroga, E. (2020), Freeze-Drying Technology in Foods, *Foods* **9**(7) 1–88.

Qua, F. J. S. (2019), A Qualitative Study on Sustainable Materials for Design Through a Comparative Review of Leather and Its Modern Alternatives, Master's thesis, Cambridge, Massachusetts Institute of Technology.

Rafiee, K., Kaur, G. and Brar, S. K. (2021), Fungal biocomposites: How process engineering affects composition and properties?, *Bioresource Technology Reports* **14**.

Raman, J., Kim, D. S., Kim, H. S., Oh, D. S. and Shin, H. J. (2022), Mycofabrication of Mycelium-Based Leather from Brown-Rot Fungi, *Journal of Fungi* **8**(3) 1–19.

Rogg, L. E., Fortwendel, J. R., Juvvadi, P. R., Lilley, A. and Steinbach, W. J. (2011), The chitin synthase genes chsA and chsC are not required for cell wall stress responses in the human pathogen Aspergillus fumigatus, *Biochemical and Biophysical Research Communications* **411**(3) 549–554.

Ruiz-Herrera, J. (2016), *Fungal Cell Wall: Structure, Synthesis, and Assembly*, Mycology, 2nd edition, CRC Press.

Ruiz-Herrera, J. and Ortiz-Castellanos, L. (2019), Cell wall glucans of fungi. A review, *Cell Surface* **5**.

Shackelford, J. F. (2015), *Introduction to materials science for engineers*, 8th edition, Davis, Pearson Higher Education, Inc.

Silverman, J., Cao, H. and Cobb, K. (2020), Development of Mushroom Mycelium Composites for Footwear Products, *Clothing and Textiles Research Journal* **38**(2) 119–133.

Simmons, E. G. (1977), Classification of some cellulase-producing Trichoderma species, *Second International Mycological Congress, Abstracts* **1000** 618.

SINGTEX Industrial CO., Ltd. (2022), *Manufacturer Functional Fabrics | Yarns | Garments | SINGTEX*, Available at: https://www.singtex.com/ (Accessed: 18.9.2022).

Sisti, L., Gioia, C., Totaro, G., Verstichel, S., Cartabia, M., Camere, S. and Celli, A. (2021), Valorization of wheat bran agro-industrial byproduct as an upgrading filler for mycelium-based composite materials, *Industrial Crops and Products* **170**.

Sivaprasad, S., Byju, S. K., Prajith, C., Shaju, J. and Rejeesh, C. R. (2021), Development of a novel mycelium bio-composite material to substitute for polystyrene in packaging applications, *Materials Today: Proceedings* **47** 5038–5044, Elsevier Ltd.

Sunagawa, N. and Igarashi, K. (2021), Expression of Recombinant Fungal Proteins in Pichia Pastoris, *Encyclopedia of Mycology* 518–527.

Sutherland, T. D., Young, J. H., Weisman, S., Hayashi, C. Y. and Merritt, D. J. (2010), Insect Silk: One Name, Many Materials, *Annual Review of Entomology* **55**(1) 171–188.

Svensson, S. E., Ferreira, J. A., Hakkarainen, M., Adolfsson, K. H. and Zamani, A. (2021), Fungal textiles: Wet spinning of fungal microfibers to produce monofilament yarns, *Sustainable Materials and Technologies* **28**.

Svensson, S. E., Oliveira, A. O., Adolfsson, K. H., Heinmaa, I., Root, A., Kondori, N., Ferreira, J. A., Hakkarainen, M. and Zamani, A. (2022), Turning food waste to antibacterial and biocompatible fungal chitin/chitosan monofilaments, *International Journal of Biological Macromolecules* **209** 618–630.

Syed, U., Parte, S. and Sayed, U. (2015), Recycling of Non Woven Waste, *International Journal of Advanced Science and Engineering (IJASE)* **1** 67–71.

Talbot, J. M., Allison, S. D. and Treseder, K. K. (2008), Decomposers in disguise: Mycorrhizal fungi as regulators of soil C dynamics in ecosystems under global change, *Functional Ecology* **22**(6) 955–963.

Tang, H., Wang, S., Wang, J., Song, M., Xu, M., Zhang, M., Shen, Y., Hou, J. and Bao, X. (2016), N-hypermannose glycosylation disruption enhances recombinant protein production by regulating secretory pathway and cell wall integrity in Saccharomyces cerevisiae, *Scientific Reports* **6**(1) 1–13.

Ullah, M., Xia, L., Xie, S. and Sun, S. (2020), CRISPR/Cas9-based genome engineering: A new breakthrough in the genetic manipulation of filamentous fungi, *Biotechnology and Applied Biochemistry* **67**(6) 835–851.

Vandelook, S., Elsacker, E., van Wylick, A., de Laet, L. and Peeters, E. (2021), Current state and future prospects of pure mycelium materials, *Fungal Biology and Biotechnology* **8**(1).

Vegatex Biotech Ltd. (2022), *About Us*, Available at: https://appleskin.com/ (Accessed: 18.9.2022).

Vegea (2022), *VEGEA: innovative biomaterials for fashion & design*, Available at: https://www.vegeacompany.com/ (Accessed: 18.9.2022).

Wang, T., Mentink-Vigier, F., Chen, A., Muszyński, A., Kirui, A., Kang, X., Widanage, M. C. D., Wang, P. and Azadi, P. (2018), Molecular architecture of fungal cell walls revealed by solid-state NMR, *Nature Communications* **9**(1) 1–12.

Ward, O. P. (2012), Production of recombinant proteins by filamentous fungi, *Biotechnology Advances* **30**(5) 1119–1139.

Weaire, D. and Hutzler, S. (1999), *The Physics of Foams*, Oxford, Oxford University Press.

Webster, J. and Weber, R. (2007), *Introduction to Fungi*, 3rd edition, New York, Cambridge University Press.

Wei, H., Wu, M., Fan, A. and Su, H. (2021), Recombinant protein production in the filamentous fungus Trichoderma, *Chinese Journal of Chemical Engineering* **30** 74–81.

Wijayarathna, E. R. K. B., Mohammadkhani, G., Soufiani, A. M., Adolfsson, K. H., Ferreira, J. A., Hakkarainen, M., Berglund, L., Heinmaa, I., Root, A. and Zamani, A. (2022), Fungal textile alternatives from bread waste with leather-like properties, *Resources, Conservation and Recycling* **179**.

Wösten, H. A. B., de Vries, O. M. H. and Wessels, J. G. H. (1993), lnterfacial Self-Assembly of a Funga1 Hydrophobin into a Hydrophobic Rodlet Layer, *The Plant Cell* **5**(11) 1567–1574.

Yoshimi, A., Miyazawa, K., Kawauchi, M. and Abe, K. (2022), Cell Wall Integrity and Its Industrial Applications in Filamentous Fungi, *Journal of Fungi* **8**(5) 1–24.

Zamani, A., Jeihanipour, A., Edebo, L., Nlklasson, C. and Taherzadeh, M. J. (2008), Determination of glucosamine and N-acetyl glucosamine in fungal cell walls, *Journal of Agricultural and Food Chemistry* **56**(18) 8314–8318.

Zhang, W., An, N., Guo, J., Wang, Z., Meng, X. and Liu, W. (2021), Influences of genetically perturbing synthesis of the typical yellow pigment on conidiation, cell wall integrity, stress tolerance, and cellulase production in Trichoderma reesei, *Journal of Microbiology* **59**(4) 426–434.