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Insights into decomposition strategies using spectroscopic and molecular approaches

Ahlawat, Ashish

2026

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Science at Lund University to be publicly defended on 13th of March at 09.00 in Blue Hall, Department of Biology, Kontaktvägen 10, Lund, Sweden

Faculty opponent
Dr. Taina Lundell

University of Helsinki

Organization: LUND UNIVERSITY

Document name: Doctoral thesis **Date of issue** 2026-03-13

Author(s): Ashish Ahlawat

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White rot fungi exhibited greater transcriptional plasticity, with cellulolytic responses strongly influenced by nitrogen availability. A comparable nitrogen effect was also observed in *Coniophora puteana*, suggesting that this species does not conform to a canonical brown rot strategy and retains functional features typically associated with white rot fungi, including the expression of cellobiohydrolases. Litter decomposers, soft rot fungi, and fungi with unresolved decay strategies showed mixed transcriptional profiles that combine conserved cellulose response genes with lineage specific regulation, further challenging strict decay type classifications. Raman spectroscopy provided a substrate centered view of cellulose modification and captured biochemical changes not reflected in gene expression alone. Raman features associated with cellulose oxidation and crystallinity were informative, with the high frequency spectral region contributing strongly to differentiation among decay trajectories. Raman signatures consistently reflected nitrogen availability and time.

Machine learning applied to integrated transcriptomic and Raman datasets improved discrimination of decay strategies and identified key molecular and spectral features underlying functional divergence. Together, these results show that white rot and brown rot strategies remain partially distinguishable on crystalline cellulose, but that their boundaries are dynamic and context dependent.

Key words: White-rot fungi, Brown-rot fungi, Raman spectroscopy, Transcriptomics,

Language: English **Number of pages**: 78

ISBN: 978-91-8104-789-9 (print)

ISBN: 978-91-8104-790-5 (electronic)

Recipient’s notes

Signature Date 2026-02-03

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Cover image by Nova Borealis Co

Published by:
Department of Biology
Faculty of Science
Lund University
Lund 2026

ISBN: 978-91-8104-789-9 (print)
ISBN: 978-91-8104-790-5 (electronic)

Printed in Sweden by Media-Tryck, Lund University,
Lund, 2026

"The map had been the first form of misdirection, for what is a map but a way of emphasizing some things and making other things invisible?"

- Jeff VanderMeer, Annihilation

Table of Contents

Abstract

Cellulose is the most abundant biopolymer on Earth and a major reservoir of organic carbon in terrestrial ecosystems. In forests, much of this cellulose originates from wood and is decomposed by saprotrophic basidiomycetes. These fungi are traditionally classified as white rot or brown rot species based on their decay mechanisms in lignocellulosic substrates. Whether these functional categories remain distinct when lignin is absent remains unresolved. In this thesis, transcriptomic and Raman spectroscopic data from fungi cultivated on crystalline cellulose are integrated to evaluate decay type differentiation under cellulose only conditions, with emphasis on nitrogen availability and temporal dynamics. Comparative transcriptomic analyses show that cellulose decay strategies form a continuum rather than discrete white rot and brown rot groups. A conserved set of genes is consistently expressed across fungi under cellulose conditions, indicating a shared transcriptional program for cellulose utilization. This conserved response is dominated by oxidative mechanisms, with widespread upregulation of lytic polysaccharide monooxygenases across all taxa. In contrast, brown rot fungi display a distinct and reproducible transcriptional signature characterized by upregulation of specific oxidoreductases, transporters, and monooxygenases lacking cellulose binding domains. This pattern indicates that, despite multiple evolutionary origins, brown rot fungi have convergently acquired similar gene networks specialized for oxidation driven disruption of crystalline cellulose.

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Popular science summary

Cellulose is the most abundant organic material on Earth and a major component of plant biomass. It forms the structural framework of plant cell walls and is therefore present in wood, leaves, grasses, and many plant-derived materials used in everyday life, such as paper, cardboard, and cotton. Large amounts of cellulose are continuously produced as plants grow across terrestrial ecosystems. Cellulose is difficult to break down because of its strong and tightly packed structure. Most organisms lack the ability to sufficiently utilize cellulose. In natural environments, this task is carried out primarily by fungi, but also by other microbes. Fungi secrete specialized enzymes and other agents that break cellulose into smaller molecules, which allows them the uptake of these molecules through absorption.

Through this process, fungi prevent the long-term accumulation of dead plant material. Without fungal decomposition, wood and plant litter would persist for extended periods, limiting soil formation and reducing the availability of nutrients for new plant growth. Over very long timescales, undecomposed plant material could become buried, altering how carbon is stored and released in the environment. Fungal decomposition of cellulose is therefore essential for maintaining healthy ecosystems. It supports the recycling of nutrients, contributes to soil development, and plays an important role in the natural carbon cycle by returning carbon from plant biomass to the soil and atmosphere.

In addition to its ecological importance, fungal cellulose degradation has practical applications. Fungi and their enzymes including cellulolytic enzymes are widely used to break down organic waste, support composting processes, and convert plant biomass into useful products. Research in this area also informs the development of sustainable technologies, including biofuel production and environmentally friendly methods for processing plant-based materials. Understanding how fungi decompose cellulose is thus important both for explaining how ecosystems function and for developing biological solutions to environmental and industrial challenges.

Populärvetenskaplig sammanfattning

Cellulosa är det vanligaste organiska materialet på jorden och en viktig komponent i växtbiomassa. Det utgör den strukturella ramen för växtcellväggar och finns därför i trä, löv, gräs och många växtbaserade material som används i vardagen, såsom papper, kartong och bomull. Stora mängder cellulosa produceras kontinuerligt när växter växer i terrestra ekosystem. Cellulosa är svår att bryta ner på grund av dess starka och tätt packade struktur. De flesta organismer saknar förmågan att utnyttja cellulosa tillräckligt. I naturliga miljöer utförs denna uppgift främst av svampar, men även av andra mikrober. Svampar utsöndrar specialiserade enzymer och andra ämnen som bryter ner cellulosa i mindre molekyler, vilket gör att de kan ta upp dessa molekyler genom absorption.

Genom denna process förhindrar svampar långsiktig ansamling av dött växtmaterial. Utan svampnedbrytning skulle trä och växtskräp finnas kvar under längre perioder, vilket begränsar jordbildningen och minskar tillgången på näringsämnen för ny växttillväxt. Under mycket långa tidsperioder skulle onedbrutet växtmaterial kunna begravas och förändra hur kol lagras och frigörs i miljön. Svampnedbrytning av cellulosa är därför avgörande för att upprätthålla friska ekosystem. Det stöder återvinning av näringsämnen, bidrar till markutveckling och spelar en viktig roll i den naturliga kolcykeln genom att återföra kol från växtbiomassa till marken och atmosfären.

Förutom sin ekologiska betydelse har svampnedbrytning av cellulosa praktiska tillämpningar. Svampar och deras enzymer, inklusive cellulolytiska enzymer, används ofta för att bryta ner organiskt avfall, stödja komposteringsprocesser och omvandla växtbiomassa till användbara produkter. Forskning inom detta område bidrar också till utvecklingen av hållbara tekniker, inklusive produktion av biobränslen och miljövänliga metoder för bearbetning av växtbaserade material. Att förstå hur svampar bryter ner cellulosa är därför viktigt både för att förklara hur ekosystem fungerar och för att utveckla biologiska lösningar på miljömässiga och industriella utmaningar.

List of papers

Paper I

Ahlawat A., Tunlid A., & Floudas D. (2025). Separating the effects of brown-rot and white-rot fungi on crystalline cellulose using Raman spectroscopy. Manuscript.

Paper II

Ahlawat A., & Floudas D. Comparative transcriptomics of saprotrophic basidiomycetes on cellulose under two different nitrogen conditions. Manuscript.

Paper III

Ahlawat A., & Floudas D. Effect of nitrogen and incubation time on cellulose decomposition by diverse saprotrophic basidiomycetes using multivariate spectral analysis. Manuscript.

Author's contributions to the papers

Paper I

AA and DF planned and designed the experiment. DF acquired the funding. AA performed the lab work and the spectroscopic measurements. AA, DF, and AT analysed the data. AA, DF and AT wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Paper II

AA and DF planned and designed the experiment. AA and DF acquired the funding. AA performed the lab work (RNA extraction). AA performed the downstream data processing and bioinformatic analysis. AA and DF wrote the manuscript. All authors contributed critically to the drafts and gave approved the final manuscript.

Paper III

AA and DF planned and designed the experiment. DF acquired the funding. AA performed the lab work and the spectroscopic measurements. AA analysed the data. AA and DF wrote the manuscript. All authors contributed critically to the drafts and gave approved the final manuscript.

AA – Ashish Ahlawat
DF- Dimitrios Floudas
AT – Anders Tunlid

Abbreviations

BR	Brown-rot
CAZY	Carbohydrate-Active Enzymes
CBH	Cellobiohydrolase
CBM	Carbohydrate binding module
CDH	Cellobiose dehydrogenases
GH	Glycoside hydrolase
LD	Litter decomposers
LPMO	Lytic polysaccharide monooxygenase
OG	Orthogroups
PLS	Partial least square
SR	Soft-rot (Ascomycete)
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl radical
UWD	Uncertain wood decay type
WR	White-rot

Introduction

Wood is the structural tissue found in the stems and roots of trees and other woody plants. It is a natural composite made of strong, tension-resistant cellulosic fibers held together by lignin and hemicelluloses, which helps the tissue resist compression (Kurei et al., 2024). For thousands of years people have used wood as fuel, for building, making tools, weapons, furniture and paper. More recently it has also become a source of purified cellulose and products such as cellophane and cellulose acetate.

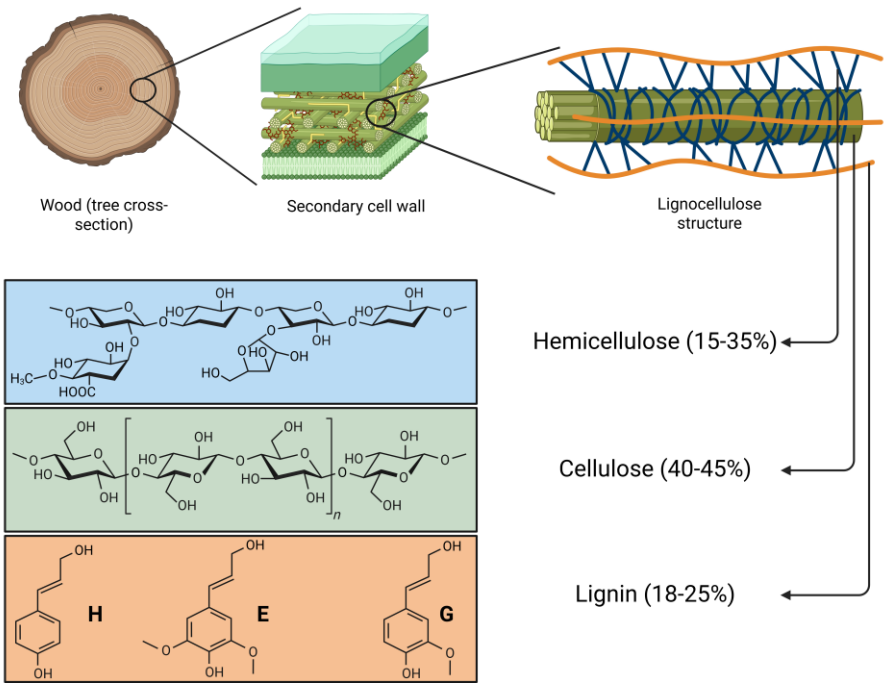

Figure 1. Visualization of wood anatomy from the tissue level to the molecular level
The cross section shows the secondary cell wall, followed by its internal architecture and the three main biopolymers: cellulose, hemicellulose and lignin, along with their characteristic structures and typical percentages in wood. Created in https://BioRender.com

Trees produce wood by adding new layers between the inner bark and the older wood. This process, known as secondary growth, gradually thickens the stem, branches and roots. The new cells form sturdy secondary walls made mainly of cellulose, hemicellulose and lignin. Although different species vary, wood is roughly 50% carbon, 42% oxygen, 6% hydrogen, 1% nitrogen and 1% other elements by weight (Onumejor et al., 2018). Wood itself is composed of three key components (Fig. 1). Cellulose makes up about 41-43% of the dry weight of wood. Hemicellulose comes next, representing about 20% of deciduous wood and nearly 30% of coniferous wood. Lignin accounts for about 27% of dry biomass in conifers and 23% in deciduous species, and its aromatic structure gives wood its water-resistant character (Rowell, 2012). These components are tightly interwoven, with chemical links between lignin and hemicellulose (Cui et al., 2022).

In 2020, forests held about 557 billion cubic meters of growing stock with wood being a major resource (FAO, 2020), and by 2023 nearly 4 billion cubic meters of wood were harvested worldwide (Branthomme et al., 2023). These large volumes feed many industries, especially construction and furniture. As a result, interest in wood as a carbon-neutral renewable resource continues to grow (Mariani and Malucelli, 2024).

Cellulose: Structure

As cellulose forms the largest share of wood and represents an energy source for organisms and the raw material in various applications, understanding cellulose itself is essential for exploring how wood is processed, transformed and used in modern materials and how it is utilized by organisms. Cellulose, the most abundant organic compound on Earth, exhibits remarkable resistance to natural decay and imparts structural strength to wood. It is a polysaccharide, existing in an ordered structure in cell wall of all green plants (Viridiplantae), other algae, tunicates, and bacteria. With approximately 1.5 trillion tons produced annually, cellulose stands as the most abundant polymer on the planet (Field et al., 1998). Its applications span across diverse industries, including paper, textiles, pharmaceuticals, among others.

Cellulose is a linear hydrophilic polymer, comprised of glucose subunits linked together through β (1→4) glycosidic linkages (Brown Jr., 2004). These chains organize into microfibrils, which, in turn, assemble into fibrils through non-covalent interactions, primarily hydrogen bonding (Nishiyama et al., 2002). Notably, the -OH group on the sixth carbon atom engages in intermolecular hydrogen bonding with other -OH groups, resulting in various types of hydrogen bonding that can lead to different cellulose structures. Consequently, cellulose is categorized into four distinct allomorphs: I, II, III, and IV (Wada et al., 2004). Cellulose in its natural

state, as a type I allomorph, is termed crystalline cellulose due to presence of crystallites with high crystallite length (40-48nm) and width (equals to number chains, 3-5 nm in plant cellulose) (Liesiene et al., 2025). The other allomorphs are generated through treatments involving harsh chemicals (Wada et al., 2004). These treatments alter the orientation of cellulose chains or disrupt hydrogen bonding, without changing the chemical structure.

Disruption of the hydrogen bonds in cellulose results in a disordered structure with its crystallinity lost and is referred to as amorphous cellulose (Ciolacu et al., 2011). This loss of crystallinity has been used to make cellulose more flexible in the paper and pulp industry. Several techniques have been used to quantify changes in crystallinity, including X-ray diffraction (XRD) (Cao and Tan, 2005; Bansal et al., 2010), nuclear magnetic resonance (NMR) (Evans et al., 1995), Fourier transform (FTIR) infrared spectroscopy (Kljun et al., 2011) and Raman spectroscopy (Agarwal et al., 2013). Some studies have also shown there is a gradient between crystalline and amorphous cellulose which affects dissolution of cellulose (Ioelovich et al., 2010). Crystalline cellulose is more abundant than amorphous cellulose in plants because of its higher tensile strength (Sixta et al., 2015) with its crystallinity around 52-60% in wood and 55-73% in cotton. Crystalline cellulose, along with lignin, plays a vital role in providing mechanical support and protection within primary and secondary cell walls of plants (Stewart and Rothwell, 1993).

Decomposition of cellulose by saprotrophic fungi

Primitive plants initially incorporated cellulose into their cell walls as a response to selection pressures such as wind and desiccation (Duchesne and Larson, 1989), providing mechanical support crucial for the development of stems and leaves (Percival and McDowell, 1981). Around 400 million years ago, the introduction of lignin, serving as a defense mechanism against predation and protecting vascular tissues, coincided with plants evolving mechanisms to create secondary cell walls (Stewart and Rothwell, 1993) with decreased nitrogen content. Evolutionary shifts that favour reallocating nitrogen away from costly cell-wall defenses and toward photosynthesis explain why plants were able to reduce nitrogen in their cell walls (Feng et al., 2009). While these organic polymers are resistant to microbial decomposition, some microbes have co-evolved with plants to extract nutrients and carbon from wood (Ayuso-Fernández et al., 2019). Many of these microbial decomposers, have developed various mechanisms to at least partially degrade polymers present in wood tissues (Álvarez et al., 2016; Singh et al., 2016). The evolution of microbial strategies to decompose cellulose reflects a long-standing biochemical arms race that has shaped terrestrial ecosystems since the rise of vascular plants (Wilson, 2008; Cornelissen et al., 2023).

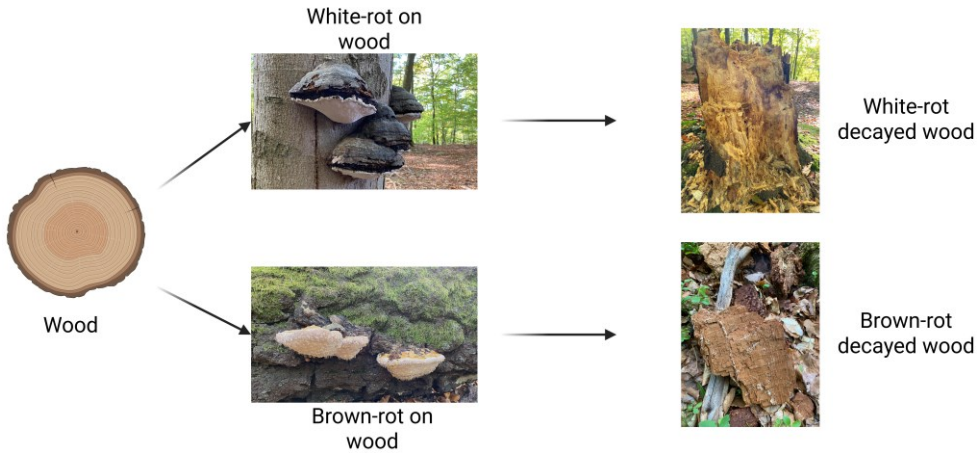

Figure 2. Two major types of wood decay
Fruit bodies of white-rot and brown-rot fungi growing on wood, and the resulting appearance of the wood after decay. Photos by D. Floudas. Created in https://BioRender.com.

Fungi can degrade crystalline cellulose and lignin in wood tissues, reducing the tensile strength and overall economic value of wood (Mori et al., 2011; Witomski et al., 2016). Cellulose-degrading fungi are broadly distributed across Dikarya, encompassing both Ascomycota and Basidiomycota (Berlemont, 2022). Within dikarya, a saprotrophic lifestyle as litter decomposers is likewise represented in both phyla (Woodward and Boddy, 2008). Most litter-decomposing fungi possess a broad enzymatic repertoire that includes cellulolytic enzymes for degrading crystalline cellulose as well as ligninolytic enzymes for modifying or breaking down lignin (Baldrian and Valášková, 2008). Despite this metabolic versatility, the nature of the substrate plays a key role in determining which fungal groups dominate decomposition (Leifheit et al., 2024; Meng et al., 2024). In the case of lignified wood, effective degradation requires specialized decay strategies that are primarily associated with wood-decaying fungi, a functional group that is largely dominated by basidiomycetes (Taylor et al., 2015). The wood-degrading activity of mushroom-forming saprotrophic fungi plays a crucial role in organic matter recycling in terrestrial ecosystems, influencing nutrient cycling and plant material decomposition (Watkinson et al., 2006; Li et al., 2022).

Saprotrophic wood-decaying fungi are broadly categorized into white-rot, and brown-rot fungi (Fig. 2) (Eaton and Hale, 1993). White-rot fungi have a unique ability to break down all the major constituents in wood including cellulose and lignin, making them highly proficient wood decomposers (Blanchette, 1995). White-rot fungi often leave significant amounts of cellulose intact resulting in distinct white cellulose remnants (Dashtban et al., 2010). The reason behind white-rot fungi leaving cellulose intact behind still lacks full understanding. Conversely,

brown-rot fungi lack the enzymes necessary for crystalline cellulose and lignin decomposition but can still utilize cellulose as a carbon source while partially modifying lignin, leaving behind a brittle, brownish lignin residue (Monrroy et al., 2011). This puzzling wood decomposition process has attracted considerable attention from researchers seeking to elucidate the underlying mechanisms.

Enzymatic pathways for cellulose decomposition by wood-decaying fungi

Fugal cellulose decomposition involves enzymes that dismantle cellulose via hydrolysis or oxidative mechanisms (Lundell et al., 2014; Andlar et al., 2018) (Fig. 3). These enzymes are classified in the CAZY database (Cantarel et al., 2009), dedicated to the analysis of genomic, structural, and biochemical data on Carbohydrate-Active Enzymes (CAZYmes). Among CAZYmes, cellobiohydrolase (CBH) is the main glycoside hydrolase (GH) targeting crystalline cellulose, spanning two GH families: GH6 (acting on the non-reducing end) and GH7 (acting on the reducing end) (Barr et al., 1996). Typically, GH6 and GH7 enzymes have CBM1 modules (carbohydrate-binding module family 1) attached to them (Christensen et al., 2019). CBM1 facilitates the attachment of enzymes to cellulose substrates, thereby enhancing the efficiency of enzymatic hydrolysis (Várnai et al., 2013). CBH of the GH6 and GH7 families degrade cellulose into cellobiose disaccharide units (Brady et al., 2015), which are then hydrolyzed into glucose by β-glucosidase (GH1, GH3, GH5, GH9, and GH30,) (Cairns and Esen, 2010). Additionally, fungi produce endoglucanase (from families like GH5, GH12, GH45 etc.), complementing CBH but primarily targeting amorphous cellulose due to its inability to bind crystalline cellulose (Nagl et al., 2022).

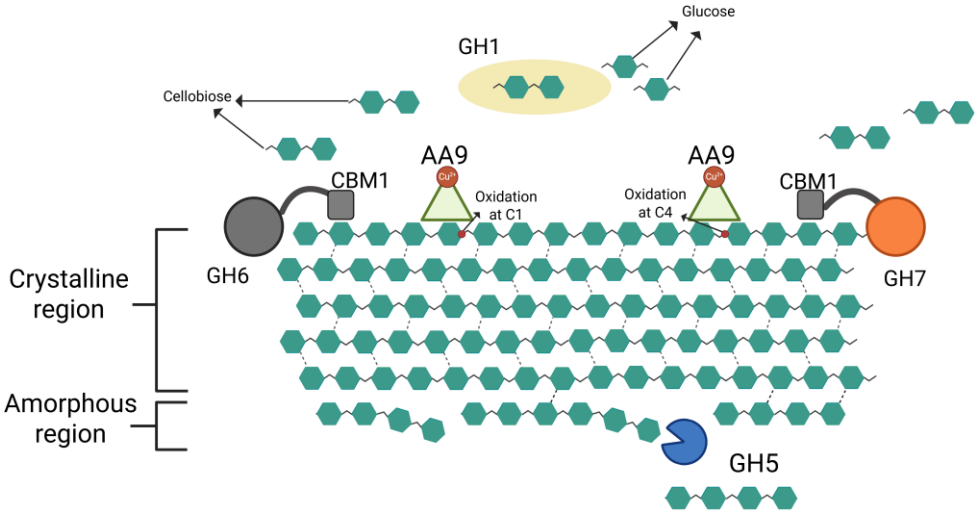

Figure 3. The fungal enzymatic decomposition of cellulose
GH5: endoglucanase, GH6: cellobiohydrolase, GH7: cellobiohydrolase, CBM1: carbohydrate-binding module family 1, AA9: LPMOs with C1-oxidation and C4-oxidation capacity, GH1: β-glucosidase. Created in https://BioRender.com.

In addition to hydrolysis, cellulose decomposition can occur through oxidative reactions. Lytic polysaccharide monooxygenases (LPMOs) represent a unique class of enzymes characterized by their mono-copper structure, specifically tailored to oxidise glycosidic bonds within cellulose (Vaaje-Kolstad et al., 2010; Bissaro et al., 2018; Munzone et al., 2024). LPMOs are categorized as Auxiliary Activities (AA) within CAZYmes, aiding in the decomposition of carbohydrates by enhancing their breakdown. Ranging from AA9 to AA17, these versatile enzymes exhibit broad substrate specificity, including cellulose, chitin, and polygalacturonic acid (Li et al., 2021; Vandhana et al., 2022). Not all LPMOs can oxidise crystalline cellulose, some LPMOs can only target amorphous cellulose (Kojima et al., 2016; Støpamo et al., 2024). Cellobiose dehydrogenase acts as the essential electron donor for LPMO, providing electrons from oxidised cellobiose (or oxygen), which activates the LPMO to perform oxidative cleavage of cellulose (Tan et al., 2015).

The enzyme repertoire differs between white-rot and brown-rot fungi (see Table 1). White-rot fungi possess CBH to degrade crystalline cellulose, whereas brown-rot fungi typically lack this enzyme. While some brown-rot fungi feature processive endoglucanase capable of targeting crystalline cellulose (Cohen et al., 2005), the exact mechanism involved in the decomposition of crystalline cellulose remains poorly understood. Nevertheless, brown-rot fungi code for most endoglucanases and β-glucosidases and they have the capacity to decompose amorphous cellulose.

Table 1. Enzymes for cellulose decomposition in wood decayers
Important enzyme classes involved in decomposition of cellulose and lignin in wood tissues and their presence across whiter-rot and brown-rot fungi (Abbreviations- WR: White rot fungi, BR: Brown rot fungi, LiP: Lignin peroxidase, MnP: Manganese peroxidase, VP: Versatile peroxidase, GH: Glycoside Hydrolase, LPMO: Lytic polysaccharide monooxygenases).

ENZYME	SUBSTRATE	PRODUCT	PRESENT IN WR	PRESENT IN BR
Cellobiohydrolase (GH6)	Crystalline cellulose (non-reducing end)	Cellobiose	Yes	No
Cellobiohydrolase (GH7)	Crystalline cellulose (reducing end)	Cellobiose	Yes	No
β-glucosidase	Cellobiose	Glucose	Yes	Yes
Endoglucanase	Amorphous cellulose	Glucose	Yes	Yes
LPMO	Crystalline and amorphous cellulose	Lactone/4-ketoaldose	Yes	Yes, (fewer gene copies)
Cellobiohydrolase (GH6)	Crystalline cellulose (non-reducing end)	Cellobiose	Yes	No

Non-enzymatic/oxidative pathways for cellulose decomposition by wood-decaying fungi

The fact that brown-rot fungi lack many of the enzymes needed for degrading lignin (Floudas et al., 2012, 2020) poses a challenge for these fungi since they must overcome the lignin barrier to reach the cellulose in the wood tissue. The limited ability of brown-rot fungi to degrade lignin has led to the development of a theory proposing an alternative mechanism that doesn't solely rely on enzymes, enabling them to bypass lignin without breaking it down. Due to the restricted pore size of wood (Tibebu and Avramidis, 2022), which restricts the passage of large proteins through the lignin matrix, it is hypothesized that small secondary metabolites may facilitate cellulose decomposition. This proposed mechanism for brown-rot fungi involves two steps (Zhang et al., 2016): first, oxidative pretreatment facilitated by metabolites and iron, and second, hydrolysis utilizing endoglucanase enzymes.

The primary theory suggests oxidation by hydroxyl radical generated through Fenton chemistry (1).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + OH^- \qquad (1)$$

In the Fenton reaction, highly reactive hydroxyl radicals (HO•) are produced by a redox reaction between soluble Fe^{2+} and H_2O_2 (Xu and Goodell, 2001; Liu et al., 2020). The HO• radicals formed through the Fenton reaction are highly oxidative, extremely short-lived and possess the ability to oxidize organic compound in their

proximity (Yu and Kuzyakov, 2021). Brown-rot fungi have been linked to the Fenton reaction, as evidenced by similar results observed when treating wood with hydroxyl radicals produced from the Fenton reaction (Kirk et al., 1991; Joseleau et al., 1994). In the literature, several pathways for producing H_2O_2 have been described, both enzymatically and non-enzymatically (using metabolites). First, oxidoreductases, such as glucose oxidase (AA3_2), aryl alcohol oxidase (AA3_2), alcohol oxidase (AA3_3) and copper radical oxidase (AA5), are known to generate H_2O_2, and these enzymes are upregulated in brown-rot fungi during wood decomposition (Zhang et al., 2016). Second, H_2O_2 can be generated by the action of redox-active metabolites that are known to be produced by brown-rot fungi (Jensen et al., 2001). Third, H_2O_2 can be produced abiotically through the oxidation of Fe^{2+} associated with ferric oxide surfaces (Jones et al., 2014).

In wood tissues, iron is mainly found in insoluble complexes with oxidation state 3 (Zelinka et al., 2021) and to participate in the Fenton reaction, the iron must be solubilized and reduced to Fe^{2+}. Numerous studies suggest that low-molecular weight (LMW) metabolites secreted by the fungi have a key role in this reduction (Table 2). According to the chelator-mediated Fenton (CMF) model (Xu and Goodell, 2001; Arantes et al., 2012; Arantes and Goodell, 2014), the hyphae of brown-rot fungi growing in the wood lumen region secretes oxalic acid that can dissolve iron oxides. At the very acidic condition in the lumen (pH ca 2.0), oxalic acid binds to Fe^{3+}, and the complexes diffuse from the lumen into the cell wall. The wood cell wall has a pH of ca 5.5. Under these conditions, oxalate has a decreased affinity for Fe^{3+} and the iron can be reduced by other LMW metabolites. The role of oxalic acid has been discussed in literature. Apart from mediating the transport of iron from the lumen to the cell wall, it has also been proposed that fungal secretion of oxalic acid is important for generating the spatial pH gradient (Arantes and Goodell, 2014). Moreover, oxalic acid has been observed to decrease the Degree of Polymerization (DOP) and viscosity of cellulose by itself (Henschen et al., 2019).

Several LMW reductants have been identified in brown-rot fungi that are thought to reduce Fe^{3+} in wood (see Table 2). Among the best characterized compounds are the quinone metabolites 2,5-DMHQ (2,5-dimethoxyhydroquinone), DMBQ (2,5-dimethoxybenzoquinone), and the related semiquinone radical. Thus, quinones are redox-active compounds that can exist in three different redox states, acting both as electron acceptor (quinone, Q), electron donor (hydroquinone (H_2Q) or as an intermediate semiquinone radical (Lyngsie et al., 2018). Notably, the DMHQ/DMBQ system can produce both reactants needed for the Fenton reaction (i.e. Fe^{2+} and H_2O_2) by driving one electron reductions of Fe^{3+} and O_2 in wood (Kerem et al., 1999; Jensen et al., 2001). The role of the quinone redox cycle for the Fenton chemistry of brown-rot fungi has mainly been studied in *Gloeophyllum trabeum*. Evidence has also been obtained that 2,5-DMHQ acts as reductant in the brown rot fungi *Serpula lacrymans* (Korripally et al., 2013) and *Postia placenta*

(Cohen et al., 2002). The fact that *G. trabeum* and *S. lacrymans* belong to two divergent lineages of basidiomycetes suggests that the 2,5-DMHQ may be prevalent in many species of brown-rot fungi. However, it should be noted that brown-rot fungi can produce several LMW metabolites that can presumably act as iron chelators and/or reductants (Goodell et al., 2006; Eastwood et al., 2011). Apart from secondary metabolites, Laccases are also found in brown-rot fungi. They are oxidoreductases and belong to the multicopper oxidase superfamily (AA1_1). Beyond their role in plant cell wall degradation, laccases participate in diverse biological processes such pigment formation, and detoxification of xenobiotics (Youn et al., 1995). The functional roles of laccases for brown-rot fungi remain to be fully understood, but they have been proposed to oxidize DMHQ (2,5-dimethoxyhydroquinone) to DMBQ (DMBQ: 2,5-dimethoxy-1,4-benzoquinone), which can subsequently reduce iron and thereby support the Fenton reaction (Wei et al., 2010).

Table 2. Extracellular enzymes or metabolites proposed to be involved in Fenton chemistry by brown rot fungi.
(DMBQ: 2,5-dimethoxy-1,4-benzoquinone, DMHQ: 2,5-dimethoxyhydroquinone; Chelator: Binds to Fe^{3+}, Reductant: Reduces Fe^{3+} to Fe^{2+})

EXTRACELLULAR SECONDARY METABOLITES	ROLE IN FENTON CHEMISTRY	REFERENCES
Oxalic acid	Chelator	(Xu and Goodell, 2001; Arantes et al., 2009)
Variegatic acid	Reductant	(Eastwood et al., 2011; Zhu et al., 2017)
Citric acid	Chelator	(Sheng et al., 2017)
2,5-DMHQ	Reductant	(Kerem et al., 1999; Jensen et al., 2001)
Glycopeptides	Reductants	(Enoki et al., 2003)

Although oxygen radicals produced by the Fenton reaction offer explanations for several phenomena, they might not fully explain the crystallinity loss seen in brown rot fungi. Due to their extremely short half-life, the effective impact of HO• radicals call for their generation near the cellulose chains, emphasizing the importance of iron localization. Instead of relying on the Fenton reaction, brown rot fungi might utilize oxidative enzymes such as LPMOs to pre-treat cellulose prior to hydrolysis.

From an evolutionary perspective, brown-rot fungi are characterized by extensive losses of genes associated with lignin decomposition. Over approximately the last 200 million years, fungi have independently evolved multiple strategies that enable efficient utilization of cellulose and hemicellulose without extensive lignin degradation, representing a clear case of convergent evolution. Genomic evidence indicates that cellulose-degrading enzymes, particularly AA9 lytic polysaccharide monooxygenases (LPMOs), were already present in ancestral fungi, whereas lignin-degrading oxidoreductases such as AA2 peroxidases evolved later (Nagy et al.,

2016). In brown-rot lineages, the loss of ligninolytic genes is thought to be associated with the emergence of alternative nutritional strategies and is considered evolutionarily irreversible. This pattern of gene loss has been documented across multiple species (Martinez et al., 2009; Eastwood et al., 2011; Floudas et al., 2012; Floudas, 2021) and underlies the evolution of the brown-rot decay strategy. Consistent with this view, brown-rot fungi are inferred to have arisen through gene loss from white-rot ancestors, and no evidence exists for a reversal from brown-rot to white-rot decay (Eastwood, 2014)

Role of nitrogen for cellulose decomposition by wood-decaying fungi

Beyond the complexity of wood structure, another challenge that fungi face during wood decay is nutrient limitation. Wood has a very high carbon-to-nitrogen (C: N) ratio sometimes reaching up to 800:1 (Rynk et al., 1992). Wood-decaying fungi, particularly white-rot fungi, rely on a diverse array of enzymes to break down polymers in wood (Baldrian, 2008). Nitrogen is needed for the synthesis of these enzymes, but also for the synthesis of fungal cell wall material including chitin. Thus, the low nitrogen content of wood likely presents a major challenge for wood-decaying fungi. Furthermore, studies have shown that fungi sense and respond to nitrogen availability. For example, it has been shown that the addition of ammonium suppresses lignin degradation in the white-rot fungus *Phanerochaete chrysosporium* (Fenn et al., 1981; Fenn and Kirk, 1981). Similar studies have not been done in brown-rot fungi. However, (Varela et al., 2003) showed that the production of enzymes and metabolites involved in the degradation of wood components by *Gloeophyllum trabeum* was reduced at lower nitrogen levels.

Nitrogen appears to be a nutrient that plays important role in altering the decomposition of fungi in general. In soil, nitrogen enrichment alters enzyme expression with enzymes linked to brown-rot decomposition (Fenton reaction) being upregulated, while those associated with white-rot decomposition (Class II peroxidases) are suppressed (Bonner et al., 2019) with some transcriptomic studies observing no effect to lignolytic enzymes and increase in abundance of cellulose-degrading genes (Xing et al., 2025). Different nitrogen sources also impact cellulose decomposition differently (Niu et al., 2025). To overcome nitrogen limitation, wood decay fungi might translocate nitrogen from soil (Clinton et al., 2009), or recycle their own chitin cell wall (Lindahl and Finlay, 2006). Despite the evidence on the role of nitrogen in fungal decomposition of the cell wall, we still have little knowledge on the differences and similarities that nitrogen may have on the decomposition processes by white-rot and brown-rot fungi.

Ecological and applied implications of fungal cellulose decomposition

Given the abundance of cellulose, microbial decomposition of cellulose is central to understanding the global carbon cycle (George et al., 2023). Fungi and bacteria drive cellulose breakdown, returning carbon to soils as organic matter and to the atmosphere as carbon dioxide, while a fraction of partially degraded material becomes incorporated into soil organic matter (SOM) (Datta, 2024). Over geological timescales, the process has contributed to soil carbon burial and coal formation, shaped in part by the evolution of plant cell wall degrading microbes (Hibbett et al., 2016). Patterns of cellulose decomposition also influence biodiversity through their effects on ecological interactions and the distribution of wood-decaying microbes in terrestrial ecosystems (Furusawa, 2019; Fukasawa, 2021). Although cellulose is rapidly decomposed and generally forms a labile pool of soil carbon, cellulose-derived carbon can persist for decades to centuries when incorporated into soil aggregates and organo-mineral associations (Miao et al., 2021). Carbon dioxide released during decomposition further provides a measurable link between cellulose turnover, greenhouse gas emissions, and climate change through ecosystem and Earth system modelling (Pendall et al., 2004).

Beyond its ecological role, cellulose represents a major renewable resource for bioenergy and biobased applications. A detailed understanding of the enzymatic processes underlying cellulose decomposition is essential for improving the efficiency of biofuel production from plant biomass (Carere et al., 2008; Antoniêto et al., 2022). Knowledge of cellulose-degrading mechanisms also supports advances in waste management and the development of more sustainable industrial processes (Chakraborty et al., 2019; Daly et al., 2021).

At the ecosystem scale, fungal cellulose decomposition governs the fate of plant-derived carbon in terrestrial environments (Deacon et al., 2006). By transforming cellulose, fungi regulate carbon redistribution among plant biomass, soil organic matter, and atmospheric carbon dioxide, thereby influencing soil formation, nutrient cycling, and long-term carbon storage (Deacon et al., 2006; Hannula and Morriën, 2022). Rather than being driven by individual enzymes alone, cellulose decomposition reflects the interaction between polymer structure, fungal metabolic capacity, and resource availability (Fukasawa and Matsukura, 2021). The degree and mode of structural modification determine both the rate of carbon turnover and the persistence of organic carbon in soils over extended timescales (Bueno et al., 2023; Rumi et al., 2024). Variation among fungal decay strategies further demonstrates that cellulose degradation proceeds through multiple biochemical pathways shaped by evolutionary history and ecological niche (Fukasawa, 2021; Hess et al., 2021).

Placing cellulose decomposition within a broader ecological and biogeochemical context highlights its relevance beyond wood decay alone. Variation in fungal decay strategies provides insight into adaptation to structurally complex, carbon-rich, and nutrient-poor substrates (Xu et al., 2025). Understanding how fungi modify cellulose structure also informs applied fields such as biomass conversion and bioenergy production, where processing efficiency depends on cellulose accessibility and organization (Michalska et al., 2012). In addition, these insights are critical for the development of sustainable cellulose-based materials, including nanocellulose, whose mechanical and functional properties are strongly influenced by biological modification of cellulose (Vigneshwaran and Satyamurthy, 2016; Nayl et al., 2025).

Aims

This thesis investigates how fungi modify crystalline cellulose and how these modifications can be detected, interpreted, and biologically contextualized by integrating chemical and molecular approaches. Rather than focusing on individual decay mechanisms in isolation, the work aims to link chemical outcomes on the substrate with underlying regulatory strategies, environmental modulation, and species-specific execution across diverse fungal lifestyles.

To address this overarching objective, the thesis was structured around the following four aims:

1. **To develop and validate a chemically anchored Raman spectroscopic framework for detecting biologically meaningful modification of crystalline cellulose (Manuscript 1).**

This aim focuses on establishing a robust methodological approach that distinguishes cellulose structural changes induced by fungi from background biological variability. Chemically defined reference states are used to guide spectral feature selection and interpretation, enabling mechanistic insight into substrate modification, particularly connected to oxidation and amorphogenesis.

2. To determine whether oxidation represents a shared but differentially executed axis of cellulose modification across fungal decay strategies (Manuscript 1-3).

This aim investigates the contribution of oxidative mechanisms to cellulose modification in white-rot and brown-rot fungi, testing whether differences between decay strategies reflect the presence of oxidation. Transcriptomic analyses are used to compare the deployment and regulation of oxidative mechanisms across decay types.

3. To assess how nitrogen availability modulates the chemical mode of fungal cellulose degradation (Manuscript 2-3).

This aim examines nitrogen as an environmental factor shaping regulatory responses and the timing of cellulose decomposition, without redefining decay strategies.

4. To evaluate whether fungal decay strategies are discrete categories or a continuum shaped by regulation (Manuscript 1-3).

This aim integrates spectroscopic and transcriptomic evidence from canonical, and atypical, species to test the validity of binary decay classifications and to characterize the diversity of fungal cellulose modification strategies.

Concept and study design

This thesis comprises two methodological approaches applied to saprotrophic basidiomycetes. The first approach investigates how fungi modify crystalline cellulose during decomposition using Raman spectroscopy The second approach examines the molecular basis of cellulose degradation through transcriptomic analyses. Together, these approaches provide complementary insights into both the nature of the modifications produced and the mechanisms underlying them.

Species included in the studies

In the different chapters, 23 species with different ecologies were included in the experiments (Fig.4). My dataset included 10 white-rot species (blue), 7 brown-rot species (brown), 3 litter decomposers (green), 2 species with uncertain decay types (Almási et al., 2019) (grey), and one soft-rot species (pink). Brown-rot species represented four independently evolved brown-rot lineages (Hibbett and Donoghue, 2001). Identification of the isolates AAJ, POS, and PHP was based on their fruiting bodies, while Trichoderma (TRE) identification at the genus level was based on culture characteristics. For all remaining species, ITS sequencing was used to determine their phylogenetic placement. For the 1st Manuscript, 4 of these species were used (BAD, TVE, GLO, FPI), for the 2nd Manuscript, 8 of the species were used (BAD, TVE, PCR, CPU, GTR, FPI, CAN, GLO) while for the last manuscript, all species were used except for GLO (FD-574SV/*Stereaceae*).

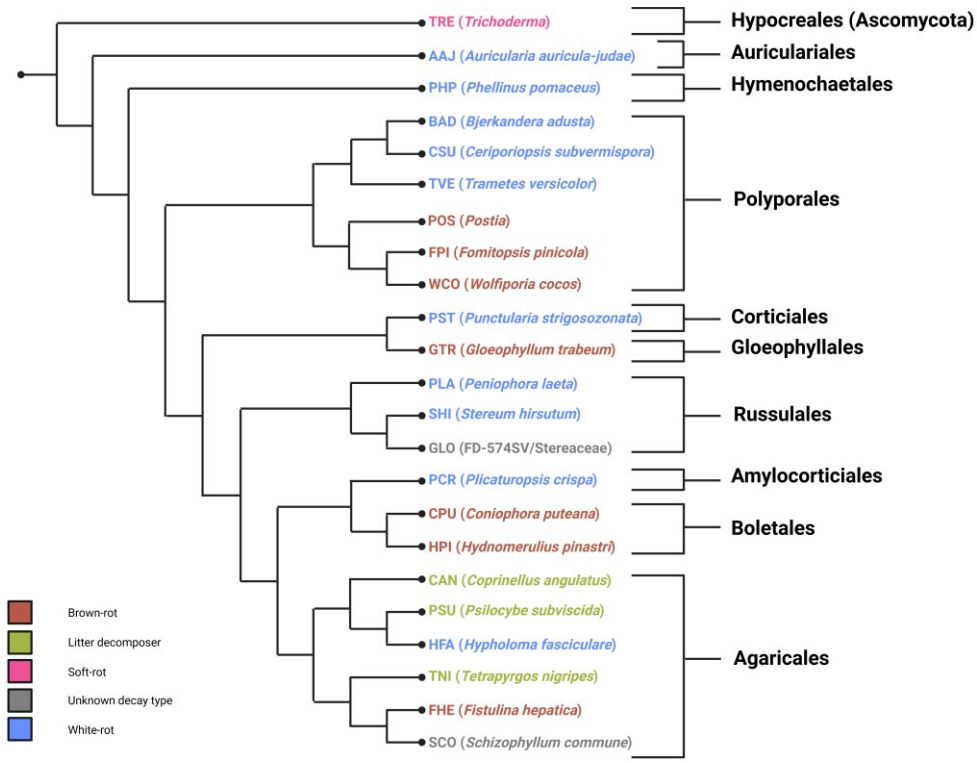

Figure 4. Phylogenetic relationships of species used in the thesis
Phylogenetic tree of the species included in the experiments based on the phylogenetic tree in Mycocosm (Grigoriev et al., 2014) with order classification and ecology of each species.

Experimental setup

Cotton (crystalline) cellulose was used as the main carbon substrate. To facilitate spectroscopic measurements, cotton pellets were prepared by using a manual hydraulic press (Fig. 5A). The set up for the spectroscopy experiments (Manuscript 1 and 3) included a single cellulose pellet on Highley media agar plates (Highley, 1973; Floudas et al., 2020) containing a low amount of glucose (0.1g/L) (Fig. 5B) with a single fungal inoculum placed next to the pellet. For the transcriptomics experiment (Manuscript 2), there were 4 pellets placed on the agar plate, with 4 fungal inocula (Fig. 5B). Glucose was added to the Highley medium to promote early growth of the strains especially for the brown-rot fungi, which tend to grow very slow when crystalline cellulose is the sole carbon source (Highley, 1973).

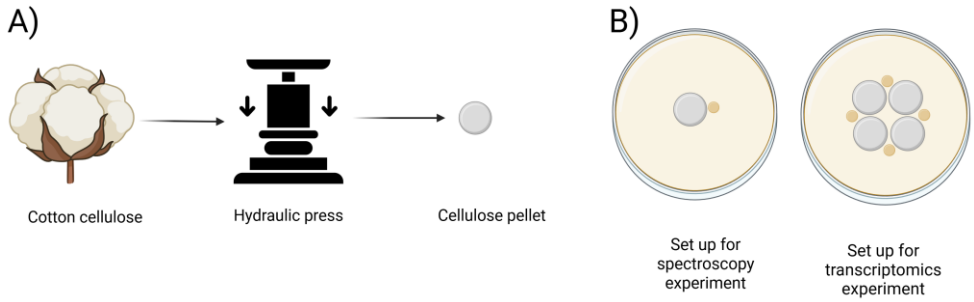

Figure 5. Preparation of the cellulose pellets and the experimental setup
A) A cotton pellet with weight of 150 mg was produced using a hydraulic press, resulting in a consistent diameter of 15 mm. B) The experimental design used either a single autoclaved pellet for spectroscopy or four autoclaved pellets for transcriptomic experiments.

Nitrogen was used as a variable with different concentrations in the media with carbon to nitrogen ratio for each condition mentioned in Table 3. These nitrogen levels were selected to investigate the fungal response to differing nitrogen concentration, with variations in the C:N ratio ranging from as high as 1200:1 to as low as 15:1. The C:N ratio of wood ranges from 200:1 to 600:1 (Onumejor et al., 2018).

Table 3. Nitrogen concentrations used in the experiment.
C:N ratio is molar ratio of carbon and nitrogen in the media plate with cellulose pellet. The calculation for the amount of carbon included both the glucose and cellulose. (M2- Manuscript 2, M3- Manuscript 3)

Condition	Nitrogen concentration (NH_4NO_3) in Highley media	C:N ratio	Remarks
HN	1 g/L	14.9:1	Used in M3
MN	0.5 g/L	29.8:1	Used in M2 & M3 (labelled as HN in M2)
LN	0.1 g/L	149:1	Used in M3
ELN	0.01 g/L	1490:1	Used in M2 & M3 (labelled as LN in M2)

Techniques

This thesis incorporates two principal techniques: Raman spectroscopy and transcriptomics. Raman spectroscopy was employed to characterise the chemical structure of both unmodified and modified cellulose, while transcriptomic analysis was used to identify the genes and gene pathways involved in fungal modification and degradation of cellulose.

Raman spectroscopy

Overview and rationale

The ordered arrangement of cellulose fibrils was discovered in 1858 (Wilkie, 1961), observations later confirmed by early X-ray diffraction studies (French and Langan, 2014). Early structural models required correction (chain packing, C6-OH orientation) and some features remained unresolved (Salem et al., 2023). Cellulose molecules assemble into crystalline fibrils whose crystal form, intermolecular interactions and higher-order organisation determine the material's physical, chemical and mechanical properties and its surface behaviour (Nishiyama et al., 2002). The crystallinity index (CI) is commonly used to quantify the relative crystalline fraction and to track changes after physicochemical or biological treatments (Agarwal et al., 2013). Although CI is usually interpreted using a two-phase model (crystalline versus amorphous), this binary view is likely oversimplified. Crystallite size, the large proportion of surface molecules in small crystallites, and continuous variation in molecular order can all influence reactivity and structure (French and Langan, 2014; Salem et al., 2023). Native cellulose typically contains less-ordered material at fibre surfaces and between crystalline regions, and this heterogeneity together with intrinsic disorder in lignocellulosic biomass complicates CI estimation by methods such as XRD, solid-state NMR, Raman and FTIR (Agarwal et al., 2013; Agarwal, 2014).

Raman spectroscopy is based on the principle of Raman scattering, where a molecule scatters light from a high-energy laser or source. Most of the scattered light remains at the same wavelength, but a small fraction (approximately 1 in 10^{-7} photon) scatters at a distinct wavelength from the source (Fig. 6). This scattered light generates a spectrum that provides insights into the chemical structure, phase and polymorphy, crystallinity, and molecular interactions of the sample (Jones et al., 2019). Each peak within the spectrum corresponds to specific chemical bond vibrations, providing insights into the sample's chemical structure without the need for extensive sample preparation. Compared to XRD, Raman spectroscopy is predominantly a surface-sensitive technique with XRD probing the full cross-section of cellulose fibrils, while Raman sampling only the near-surface region, with an effective penetration depth of approximately 2-6 μm (Everall et al., 2007). As a result, XRD provides more detailed information on structural spacing and lattice-level changes within cellulose, whereas Raman spectroscopy is better suited for detecting chemical modifications at or near the surface (Stacey et al., 2021).

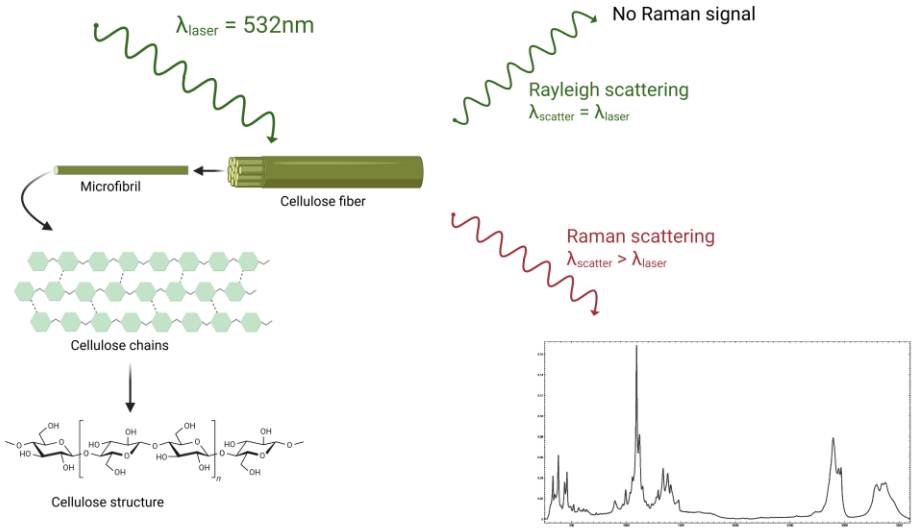

Figure 6. Conceptual overview of Raman scattering and its application to cellulose.
Raman scattering occurs when the wavelength of a laser changes after interacting with a chemical bond The cellulose examined here is a carbon-based substrate made of glucose chains linked by glycosidic bonds, which assemble into microfibrils and then into cellulose fibers. The spectrum shown represents a Raman signal collected from a single carbon fiber.

Methodological applications of spectroscopy in fungal decomposition research

Raman spectroscopy is an established method for assessing the structural organisation of cellulose in both its native and modified states (Agarwal, 2022). These studies have offered valuable insights in vibrations that are informative about the higher structure and chemistry of cellulose (Wiley and Atalla, 1987). Existing work has predominantly addressed physical and chemical alterations, with a particular focus on crystallinity. However, analysis of cellulose oxidation using Raman spectroscopy is largely unexplored (Agarwal et al., 2010; Agarwal, 2019; Floudas et al., 2020). Fungal modification of crystalline cellulose has been investigated only once, and that study examined a restricted spectral window, leaving most Raman-active structural changes unassessed (Floudas et al., 2020).

Raman analysis of modified cellulose is complicated by multiple vibrational modes responding at once to changes in bonding, conformation, and supramolecular structure. In addition, the intensity of several cellulose bands is strongly influenced by fibril orientation relative to the laser, making it difficult to separate orientation effects from chemical or structural modifications (Wiley and Atalla, 1987).

Analyses involving fungal material introduce further complications with organic compounds contributing substantial autofluorescence (Cordero et al., 2018). Fungal pigments and extracellular enzymes can produce Raman bands that overlap those of cellulose (Lu et al., 2018), thereby reducing spectral differentiation and making analysis complicated. Along with this, higher acquisition time make measurements time consuming.

Data acquisition and analysis

Raman spectrometer with green laser with a wavelength of 532nm was used with measurements done in six spectral regions (60-250 cm^{-1}, 250-600 cm^{-1}, 820-945 cm^{-1}, 945-1225 cm^{-1}, 1225-1495 cm^{-1}, and 2800-3600 cm^{-1}). The acquisition time (time of exposure) was 7 seconds with accumulation (total spectra) also 7. All spectra, after measurements, were baseline corrected and normalised (vector) using Quasar (Orange spectroscopy) (Toplak et al., 2021). The iPeak script in MATLAB (O'haver, 1991) was used for peak identification, and OCTAVVS (Troein et al., 2020) was employed to integrate spectral files from multiple experimental conditions into a unified dataset for downstream analysis. R Statistical Software in R studio was used for creating reference libraries (Manuscript 1) and visualisation. Peak fitting, PCA and machine learning algorithms (Random Forest, PLS) were also widgets used from Quasar (Manuscript 3).

Transcriptomics

Overview and rationale

Transcriptomics measures gene expression in a sample at a defined time point. Because transcriptional changes frequently represent the earliest molecular responses to environmental or physiological cues, transcriptome profiling provides a direct view of how organisms control metabolic pathways, stress responses, and developmental processes (Kittleson et al., 2009). In microbial research, transcriptomics is particularly suited to identify genes involved in substrate degradation, nutrient acquisition, and ecological specialisation (Plunk et al., 2022). Comparing expression profiles across conditions allows detection of coordinated gene responses, inference of functional pathways, and formulation of hypotheses about underlying regulatory mechanisms (Kittleson et al., 2009). Together, these attributes make transcriptomics a powerful method for characterising the molecular basis of behaviour and responses to external stimuli. Transcriptomics has also become a key tool for dissecting the molecular mechanisms of fungal wood decay, facilitating identification of genes, and regulatory networks involved in breakdown of wood (Zhang et al., 2019; Min et al., 2023).

Methodological applications of spectroscopy in fungal decomposition research

Transcriptomic studies show that wood-decaying fungi use fundamentally different strategies for cellulose degradation, with major differences between white-rot and brown-rot species (Presley et al., 2018; Zhang et al., 2019). White-rot fungi, for example *Phanerochaete chrysosporium, Dichomitus squalens, Phlebia radiata* and *Schizophyllum commune*, employ a broad hydrolytic strategy using diverse glycoside hydrolases including cellobiohydrolases (GH6, GH7), β-glucosidases (GH1, GH3, GH9, GH30), endoglucanases (GH5, GH12, GH45 etc.) and AA9 lytic polysaccharide monooxygenases (LPMOs) (Martinez et al., 2009; Wymelenberg et al., 2010; Gaskell et al., 2016; Kuuskeri et al., 2016). These enzymes act synergistically to target both cellulose and lignin. By contrast, brown-rot fungi such as *Postia placenta, Fomitopsis pinicola* and *Wolfiporia cocos* rely mainly on oxidative mechanisms, notably Fenton chemistry, and show upregulation of genes for iron acquisition, redox cycling, and peroxide generation while expressing endoglucanases (Martinez et al., 2009; Wymelenberg et al., 2010; Gaskell et al., 2016; Shah et al., 2018). The enzymatic toolkit for cellulose degradation spans multiple CAZyme families together with auxiliary enzymes such as AA9 LPMOs, cellobiose dehydrogenases (CDH), and other oxidoreductases Transcriptomic data indicate that AA9 LPMO expression differs markedly among brown-rot fungi, with species showing variable levels and patterns of induction (Umezawa et al., 2020). These enzymes are often co-expressed with uncharacterised secreted proteins, implying complex interactions that enhance degradation efficiency. Gene expression is coordinated by regulatory networks involving multiple, interdependent genes and transcription factors. These factors integrate environmental signals to control expression of several CAZYmes including LPMOs (Marian et al., 2022). The substantial investment in CAZymes production underscores their importance in nitrogen-limited wood substrates, however, transcriptomic approaches have not been applied yet to examine how wood-decaying fungi respond to nitrogen availability.

Methodologically, high-throughput RNA-seq enable genome-wide profiling of fungal expression during wood decomposition. Integrating transcriptomic and secretomic data has been especially informative for revealing enzyme and regulatory coordination (Martinez et al., 2009; Presley et al., 2018), although purity of extracted RNA is decreased with recalcitrant wood substrates. Despite progress, many upregulated genes, particularly low-abundance CAZymes and unknown secreted proteins, lack functional characterisation despite evidence for specialised roles in substrate specificity and enzyme interactions. The regulatory networks controlling genes for crystalline cellulose degradation remain incomplete, and current studies using either wood or Avicel (chemically created crystalline cellulose) in liquid cultures may not fully capture fungal strategies for attacking

native crystalline cellulose or the complexity of natural wood decay with nitrogen affecting the fungal responses (Fenn et al., 1981).

Data acquisition and analysis

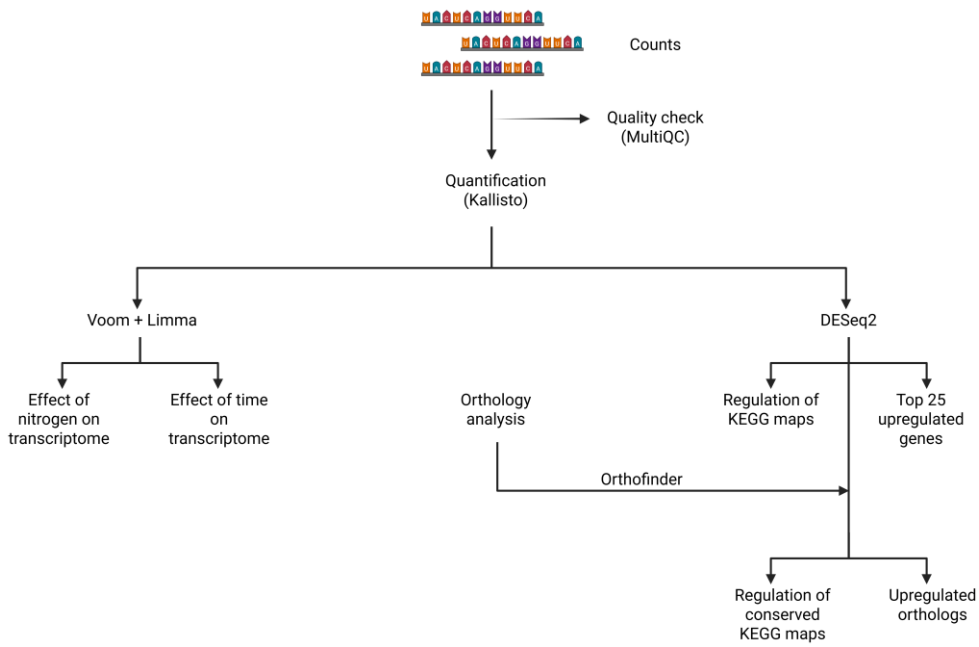

Figure 7. Workflow for the transcriptomics experiment.
Raw counts were obtained from BGI Genomics, and kallisto was used to generate transcript abundances through pseudoalignment to the reference genome. For FD-574SV (*Stereaceae*), no reference genome was available, so the genome of *Stereum hirsutum* was used instead. Voom + limma was applied to evaluate the independent effects of nitrogen and time, while DESeq2 was used to generate contrasts against the control (glucose) samples. Orthologs identified by OrthoFinder were used to determine conserved orthologs across all fungi as well as conserved pathways associated with each ecological group.

Transcriptomics was performed to check the response of fungi with different ecologies on how they approach crystalline cellulose. Eight species of basidiomycetes were used (Manuscript 2), and RNA was extracted using RNeasy Plant Mini Kit from Qiagen and extracted RNA was sequenced at BGI genomics. Kallisto was used to quantify transcript abundances from cDNA libraries obtained from JGI (FD-574SV, cDNA library of *Stereum hirsutum* was used, which is one of most closely related sequenced species to the strain we used in the study) (Fig. 7). The resulting abundance/count matrices were carried forward into two complementary analysis paths. Voom+limma modelled the effects of nitrogen and

time across all samples to estimate the proportion of genes significantly regulated by these factors, providing a multi-factor view of treatment effects. DESeq2 was run separately for each species; PCA was used to visualise overall species responses, and gene-level log-fold changes (LFC) and their standard errors (LFCse) were extracted for pathway-level aggregation. Gene LFCs were aggregated into pathway scores as weighted mean LFCs, and Stouffer's method was applied to combine directional evidence so that both magnitude and regulation were preserved at the pathway level. An ortholog-centric comparative analysis restricted to genes present in decay groups was used to identify conserved pathway responses and compare how the same genes and pathways respond across white-rot and brown-rot fungi. Orthologous CAZymes and other cellulose-decomposition related genes were examined for their regulation and group patterns. Finally, for each fungus a non-orthologous top 25 upregulated gene list was compiled to capture species-specific strong responders.

Main findings

In this section, I present results from the three chapters, integrating data from spectroscopy and transcriptomics. This thesis aimed to determine whether fungal modification of cellulose can be detected, classified, and biologically interpreted using Raman spectroscopy, and whether transcriptomic data can explain the observed spectroscopic patterns. This section first summaries insights from a controlled subset of species, then evaluates their generalisation across a broader phylogenetic range, and finally integrates transcriptomic evidence to interpret spectroscopic signals.

Cellulose modification by chemical treatments

To determine whether Raman spectroscopy can capture biologically meaningful modifications of crystalline cellulose, I first established a chemically defined reference framework against which fungal-induced changes could be interpreted. Raman spectroscopy detects molecular vibrations within its fingerprint region (250-1500 cm^{-1}), which contains numerous signals associated with cellulose-specific chemical bonds. Structural modifications of cellulose have previously been examined in this region to identify characteristic peaks that distinguish cellulose from other polysaccharides (Agarwal, 2019). However, such modifications are typically induced by harsh chemical treatments, such as strong alkali or acid exposure, and are most often applied during cellulose isolation from wood rather than used as analytical references. In this study, these chemical perturbations were instead applied to generate controlled modification states of cellulose.

Crystalline cellulose was treated with sodium hydroxide to induce a reduction in crystallinity and with TEMPO to selectively oxidise the C6-hydroxyl group of glucose units. TEMPO oxidation has been widely used to produce oxidised cellulose nanofibers, and its reported inability to substantially alter cellulose crystallinity made it particularly suitable for disentangling oxidation effects from amorphogenesis. Together, these treatments provided two chemical modification states. Raman measurements of biologically colonised cellulose are strongly influenced by autofluorescence originating from fungal pigments and other biological compounds, as well as by orientation effects that alter peak intensities depending on fiber alignment relative to the laser.

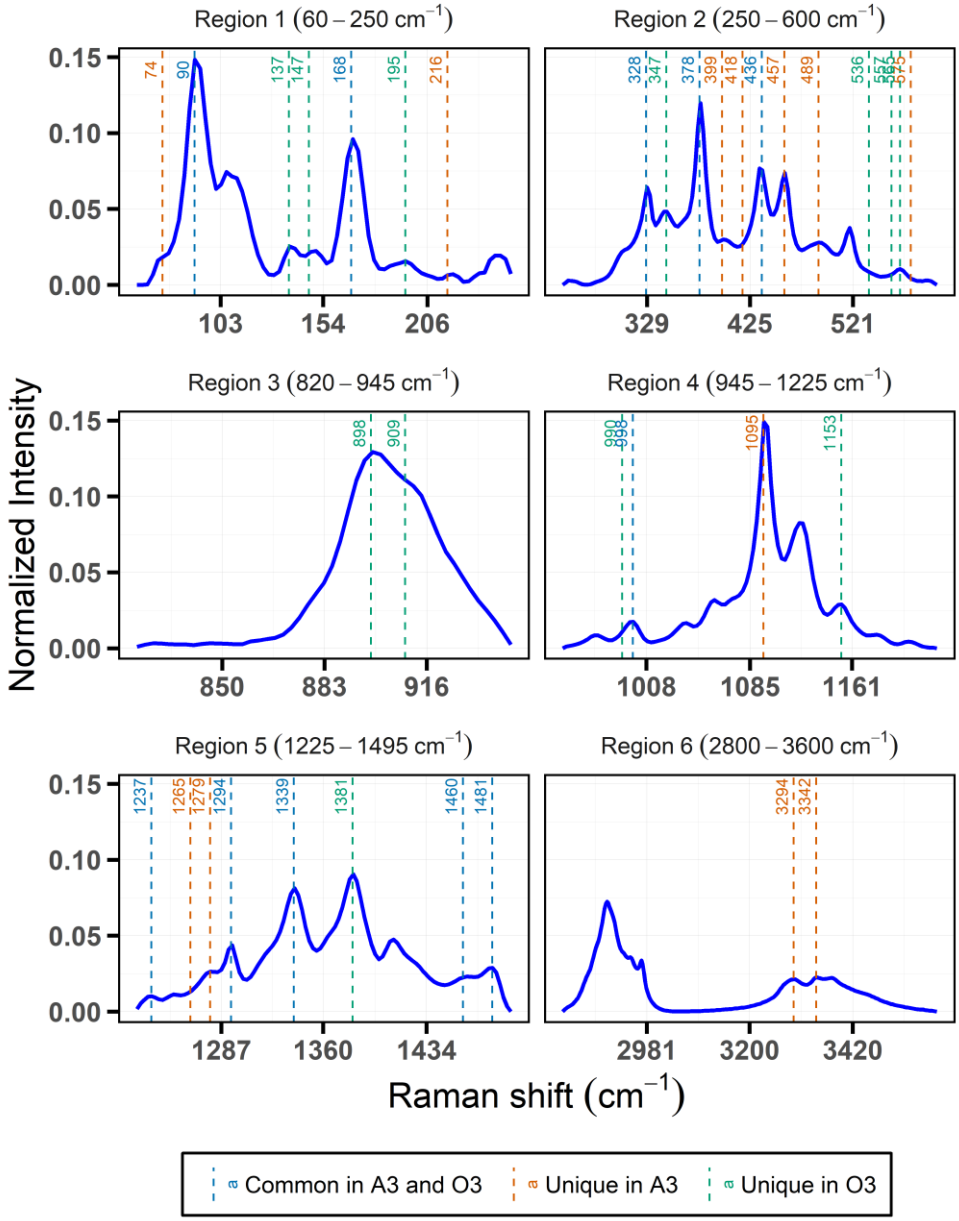

Figure 8. Raman spectra of unmodified cellulose with peak associated with amorphogenesis and oxidation highlighted.

The plot shows Raman spectra separated into six different regions. The average spectrum of crystalline cellulose (control, n=12) is shown in blue. Wavenumbers were separated into three datasets: A*, Amorphogenesis-specific wavenumbers (unique for the A3 library, indicated in green); O*, Oxidation-specific (unique for the O3 library, indicated in blue); and C*, Common wavenumbers that are present in both A3 and O3 (orange).

To minimise these sources of variability, spectra were not recorded across a single continuous range. Instead, measurements were collected from six discrete spectral regions selected specifically for their association with cellulose structure. This approach reduced background interference while retaining chemically informative signals.

Comparisons between chemically modified cellulose (oxidised and amorphous) and unmodified crystalline cellulose enabled the identification of wavenumbers associated with these specific structural changes (Fig. 8). Amorphogenesis and oxidation-related spectral variation could each be captured by a small subset of 23 wavenumbers, with 11 shared between the two, indicating partial overlap in their spectral signatures. Out of these 35 peaks, 32 have previously been attributed to cellulose structure (Table 4) with oxidation of cellulose not studied with Raman spectroscopy before. While many of these peaks have been individually reported, their coordinated response to controlled oxidation and amorphogenesis, and their combined use as a reduced and functionally interpretable spectral feature set, had not been systematically evaluated. This analysis therefore established a chemically grounded spectroscopic phenotype of cellulose modification that could be used to investigate fungal decay.

Table 4. Peaks in the Raman spectra of cellulose identified to be associated with amorphogenesis and oxidation.

The wavenumbers were grouped into three groups: A*, Amorphogenesis-specific (unique for the A3 library); O*, Oxidation-specific (unique for the O3 library); and C*, Common to both A3 and O3. Shown are proposed vibrations and information on peaks that have previously been identified to be affected by chemical treatments of crystalline cellulose or been assigned in Raman spectra of cellulose. Peaks without references suggest novel peaks assignment to oxidised cellulose.

Peaks (wavenumbers)	Vibrations	Chemical correlation	References
A* (Amorphogenesis)			
74 cm^{-1}	Crystal lattice mode	Correlated to Amorphogenesis	Agarwal et al., 2018
216 cm^{-1}		Correlated to Amorphogenesis	Agarwal et al., 2014
399 cm^{-1}			Agarwal et al., 2014
418 cm^{-1}		Correlated to Amorphogenesis	Agarwal et al., 2014
457 cm^{-1}	(CH), ξ; (CH_2), ρ		Atalla et al., 1987
489 cm^{-1}			Agarwal et al., 1997
575 cm^{-1}		Correlated to Amorphogenesis	Agarwal et al., 2014
1095 cm^{-1}	C-C and C-O, *v*; (COC) glycosidic asymmetric, *v*	Correlated to Amorphogenesis	Atalla et al., 1987
1265 cm^{-1}		Correlated to Amorphogenesis	Agarwal et al., 2021
1279 cm^{-1}			Atalla et al., 1987
3294 cm^{-1}	(OH), *v*		Atalla et al., 1987

3342 cm^{-1}	(OH), *v*		Atalla et al., 1987
O* (Oxidation)			
137 cm^{-1}			
147 cm^{-1}			Agarwal et al., 2018
195 cm^{-1}		Correlated to Amorphogenesis	Agarwal et al., 2010
347 cm^{-1}	Heavy atom, *v*		Manciu et al., 2014
536 cm^{-1}			
557 cm^{-1}			
565 cm^{-1}	(COC) ring deformation		Agarwal et al., 2014
898 cm^{-1}	(CH_2), ρ		Agarwal et al., 2021
909 cm^{-1}	HCC and HCO, *β*; (COC) in-plane symmetric, *v*		Agarwal et al., 2014
990 cm^{-1}			Agarwal et al., 2014
1153 cm^{-1}	CC and CO ring asymmetric, ν		Atalla et al., 1987
1381 cm^{-1}	HCC, HCO and HOC, *β*; (CH_2), δ		Atalla et al., 1987
C* (Common)			
90 cm^{-1}	Crystal lattice mode	Correlated to Amorphogenesis	Agarwal et al., 2018
168 cm^{-1}		Correlated to Amorphogenesis	Agarwal et al., 2022
328 cm^{-1}	Heavy atom, *β*		Atalla et al., 1987
378 cm^{-1}	(CCC) ring, δ		Manciu et al., 2014
436 cm^{-1}	(COC), ξ; (OH), γ		Manciu et al., 2014
998 cm^{-1}			Atalla et al., 1987
1237 cm^{-1}			Agarwal et al., 2014
1294 cm^{-1}	HCC and HCO, *v*; CH and ξ OHO, ξ		Atalla et al., 1987
1339 cm^{-1}			Atalla et al., 1987
1460 cm^{-1}	HCH and HOC, *β*; (CH_2), δ	Correlated to Amorphogenesis	Schenzel et al., 2005
1481 cm^{-1}	HCH and HOC, *β*; (CH_2), δ	Correlated to Amorphogenesis	Schenzel et al., 2005

Spectroscopic phenotype of decay type

Having established a spectroscopic phenotype of cellulose modification, the next step was to determine which types of structural change this phenotype captures most strongly and how this sensitivity relates to fungal decay strategies. Analysis of the reduced Raman feature set showed that oxidation-associated wavenumbers contributed most strongly to the separation between fungal decay types (Fig. 9A). When spectra were examined using subsets of features linked to specific

modification modes, oxidation-related signals consistently provided statistically significant difference between white-rot and brown-rot fungi (Fig. 9B). In contrast, wavenumbers associated primarily with amorphogenesis, or loss of crystallinity displayed a more limited ability to discriminate between decay types, despite their clear response in chemically treated reference samples. This difference in discriminatory power indicates that the fungal decomposition of cellulose is not dominated by generalised disruption of cellulose structure. Instead, it reflects chemical modifications associated with oxidative processes. Although both white-rot and brown-rot fungi alter cellulose, the oxidation-related changes they produce differ sufficiently to generate distinct spectral signatures, whereas crystallinity-related changes alone were more variable and less decay-type specific under the conditions examined.

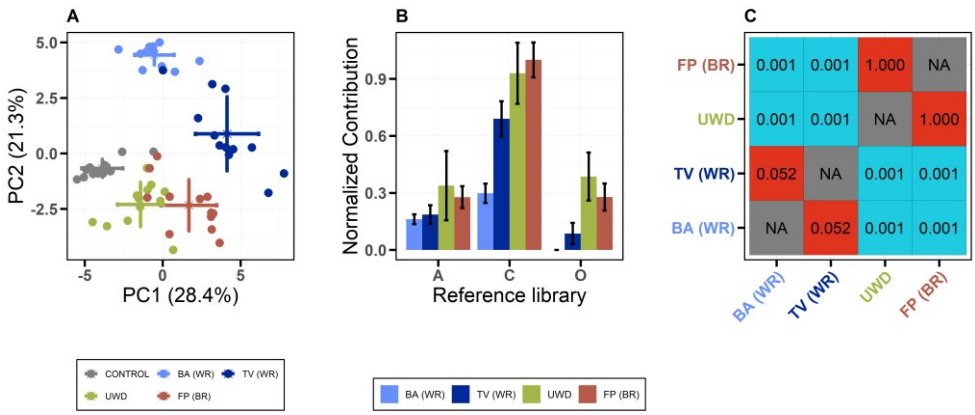

Figure 9. Raman spectroscopy of fungal-decomposed cellulose from manuscript 1.
(A) PCA plot of reduced spectral data from Raman spectra colonized by four different fungal species and untreated cellulose. In total, 35 wavenumbers in the A*, O*, and C* libraries were analyzed. Each point represents data from one sample, with each treatment carried out with 12 replicates. The cross indicates the standard deviation across PC1 and PC2 scores. (B) Contribution of wavenumbers from the A*, O*, and C* libraries for separating spectra from the four fungal species. Euclidean distances between fungal-treated and non-treated cellulose samples were calculated using the scores from PC1 and PC2, and the contribution of a given wavelength to the separation of the samples was estimated by recording the change in distance before and after removing the wavelength from the distance analysis. Values are normalized to the value representing the largest decrease in distance. Error bars represent standard deviation across replicates (n=12). (C) Pairwise comparison of spectral data from fungi-decomposed cellulose. In the heatmap, red indicates no significant differences (p-value ≥ 0.05), while blue indicates significant differences (p-value < 0.05). WR, White-rot fungi; BR, Brown-rot fungi;

The behaviour of the strain with an uncertain decay type further reinforced this pattern. Across analyses, cellulose modified by this *Stereaceae* isolate clustered more closely with brown-rot fungi than with white-rot fungi, particularly when oxidation-associated features were emphasised. This clustering was consistent despite the taxonomic placement of the strain and the presence of genes typically associated with white-rot fungi, which we detected in Manuscript 2, indicating that

the spectroscopic phenotype reflects the chemical outcome of cellulose modification rather than decay strategy inferred from taxonomy or enzymatic potential alone. Taken together, these results demonstrate that the Raman-based phenotype of decay type is especially sensitive to oxidation-driven modification of cellulose and captures a chemical axis of decay that is partly independent of traditional classifications based on genomic data. This sensitivity explains the strong separation observed in controlled comparisons, while also suggesting that intermediate or ambiguous phenotypes may emerge when a broader range of fungal strategies is considered.

Scaling the spectroscopic subset

Scaling the spectroscopic analysis to a broader set of fungal species revealed clear limitations of peak-based multivariate models for separating decay strategies. While chemically informed Raman features and PLS-based models performed well in a reduced species subset, their classification accuracy declined as additional taxa were included.

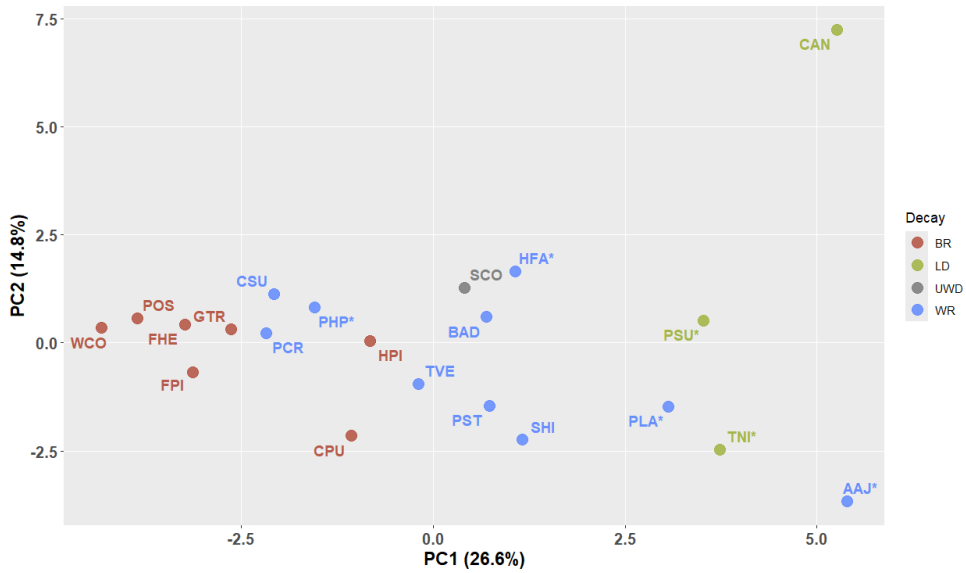

Figure 10. Principal component analysis (PCA) of CAZY enzyme profiles associated with cellulose degradation for selected fungal species used in Manuscript 3.
All CAZy data were obtained from MycoCosm (Grigoriev et al., 2014). The control represents unmodified cellulose. An asterisk (*) indicates that the data correspond to a different strain or a closely related species, rather than the exact strain used in this study.

Notably, oxidation-associated peaks that contributed to separation in smaller datasets no longer discriminated between white-rot and brown-rot fungi when the analysis was extended across greater phylogenetic diversity. The effect of expanded taxonomic coverage is reflected in the CAZyme profiles (Fig. 10), which show substantial heterogeneity in carbohydrate-active enzyme repertoires both within and across decay categories. Species assigned to the same decay type often differed markedly in their complements of glycoside hydrolases, auxiliary activity enzymes, and oxidative systems, reflecting divergent evolutionary histories and ecological specializations. This enzymatic diversity coincides with the reduced performance of peak-based Raman models, suggesting that oxidation-related spectral features informative in limited species sets are not consistently associated with decay type at larger biological scales.

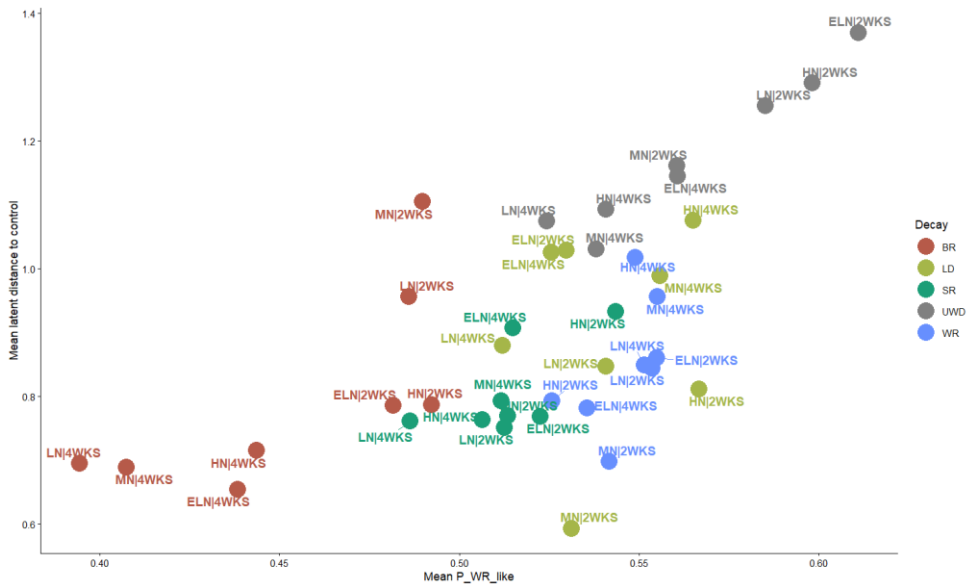

Figure. 11. Direction versus magnitude of cellulose modification across decay strategies. Scatter plot showing mean P_WR_like (position along the WR–BR axis) versus mean latent-space distance to control (overall magnitude of modification) for each decay type under different nitrogen and time conditions. This figure demonstrates that similarity to WR-like spectral patterns can occur independently of the overall extent of divergence from unmodified cellulose

Consistent with this observation, PLS-based classification models showed reduced accuracy when applied to the expanded dataset, indicating that a limited number of predefined Raman features was insufficient to capture decay-related variation across diverse taxa (Fig. 11). This study emphasizes that comprehensive sampling of species within decay categories is essential for understanding decay diversity, in contrast to approaches based on few or model organisms. In contrast, autoencoder-

based analysis of the full Raman spectra retained strong discriminative performance despite increased taxonomic and functional complexity. By incorporating distributed spectral information rather than relying on selected peaks, the autoencoder separated decay strategies across species drawn from multiple clades (Fig. 11). Soft rot (SR) or uncertain wood decayer (UWD) occupied intermediate positions in latent space rather than clustering tightly with either white-rot or brown-rot fungi. Because soft-rot and unknown wood decay fungi were each represented by one species, conclusions for these groups should be interpreted cautiously. These placements occurred despite the presence of oxidation-associated Raman features, indicating that such features alone were insufficient for decay-type separation when broader biological diversity was considered.

Atypical and intermediate species reveal decay strategies as a continuum

To examine how non-canonical decay strategies relate to canonical white-rot (WR) and brown-rot (BR) fungi, we projected Raman spectra from litter decomposers (LD), a soft-rot (SR) fungus, and an unknown wood decay (UWD) fungus onto a WR-BR reference space learned using a semi-supervised autoencoder and a regression model applied to the latent representation. Both approaches yielded broadly consistent organization of spectra along a continuous chemical axis but differed in how strongly non-canonical fungi aligned with WR-like organization. The SSAE-derived probabilities captured similarity to learned spectral structure and tended to place some non-canonical fungi closer to WR-like regions (Fig. 12), whereas the regression-derived WR-likeness provided a more conservative and stable ordering along the dominant WR-BR dimension (Fig. 7 from manuscript 3). Importantly, both models were trained using spectra from all nitrogen and time conditions, meaning that nitrogen and time effects are incorporated into the learned representation; as a result, variation associated with nitrogen and time is attenuated in the projections and should be interpreted as relative modulation rather than as an absence of nitrogen and time influence.

When WR-likeness is interpreted together with latent distance to control, distinct patterns emerge for LD, SR, and UWD (Figure 8, Manuscript 3). Litter decomposers consistently occupied WR-like positions along the chemical axis, but showed substantial divergence from the unmodified control, indicating extensive cellulose modification that aligns with WR-like organization. Soft-rot fungus (*Trichoderma*) occupied an intermediate WR-likeness range and showed moderate divergence from control, consistent with partial but constrained cellulose modification. In contrast, the UWD fungus (*S. commune*) exhibited among the highest WR-likeness values while also showing the largest distances to control, indicating strong cellulose

restructuring that nevertheless does not fully overlap with canonical WR behaviour. Species-level analyses further revealed that non-canonical fungi differ markedly in their sensitivity to nitrogen and time: some LD species showed strong condition-dependent shifts in WR-likeness and modification magnitude, while SR remained relatively stable. UWD showed substantial nitrogen and time dependent variation in both WR-likeness and divergence from control. Together, these results demonstrate that WR-likeness and extent of modification capture complementary aspects of decay behaviour and that non-canonical strategies cannot be reduced to a single intermediate state.

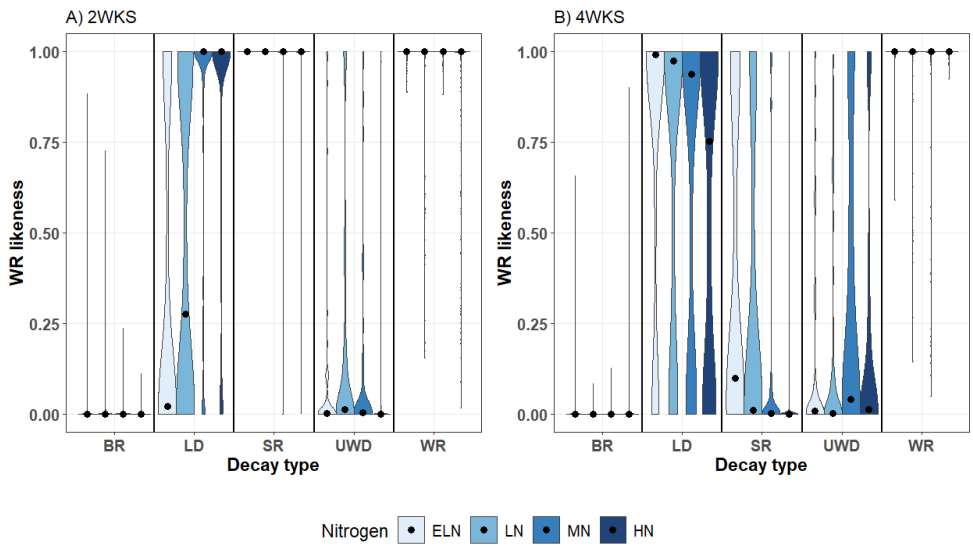

Figure 12. Decay-type distributions of autoencoder-derived (SSAE) WR-likeness scores for both time points.
Violin plots show the distribution of per-spectrum probabilistic white-rot–likeness scores (P_WR_like) generated by the semi-supervised autoencoder for each decay type. Distributions are shown separately for nitrogen conditions (color-coded) and faceted by incubation time. Embedded boxplots indicate the median and interquartile range, with whiskers representing the data spread. Each violin summarizes all individual spectra within a decay type, highlighting both within- and between-group variability. P_WR_like values range from 0 (brown-rot–like) to 1 (white-rot–like), allowing comparison of decay-related chemical phenotypes across nutrient and temporal conditions.

Overall, these findings support a view of fungal cellulose modification as a structured continuum rather than a set of discrete decay classes. WR-likeness reflects similarity in chemical organization, while distance to control captures the magnitude of structural change, and both are required to interpret decay outcomes meaningfully. Differences between SSAE-derived and regression-derived projections highlight that WR-likeness is not a uniquely defined quantity but

depends on whether similarity is assessed relative to learned spectral structure or constrained along a dominant axis. At the same time, the results should not be interpreted as direct evidence for specific biochemical mechanisms or enzymatic pathways, nor as proof that nitrogen and time are unimportant; rather, they indicate that nitrogen and time shape cellulose modification within decay-specific chemical landscapes that remain detectable even after integrating spectra across conditions.

Time and nitrogen may alter the outcome of decomposition of cellulose

The interaction between nitrogen availability and temporal cellulose modification reveals pronounced decay-type dependent patterns in both the extent and mode of chemical change. Comparisons between 2-week and 4-week incubations showed strong nitrogen and decay-type specific differences in how cellulose chemistry evolved over time. White-rot fungi exhibited the largest number of significantly altered Raman wavenumbers across nitrogen conditions, indicating sustained and chemically diverse interaction with cellulose throughout the experimental period (Manuscript 3, Tables S1-S3). These temporal changes persisted even under nitrogen limitation, demonstrating continued metabolic engagement with the substrate (Wang et al., 2014; Agarwal et al., 2018). Transcriptomic analyses further indicate that carbon-driven responses to cellulose are broadly conserved across fungi, whereas nitrogen-dependent regulation is more pronounced in white-rot fungi than in brown-rot fungi. However, nitrogen availability strongly influenced which chemical modification modes became detectable over time, rather than uniformly affecting the overall magnitude of cellulose modification. Together, these results suggest that nitrogen availability in white-rot fungi primarily modulates how cellulose modification is executed over time, rather than whether modification occurs.

Autoencoder-based classification identified the low-frequency Raman region below 120 cm^{-1} as particularly informative for distinguishing white-rot and brown-rot fungi (see Manuscript 3). This spectral region is commonly associated with collective lattice vibrations and long-range structural organization in crystalline cellulose, indicating that decay-related differences are expressed at the level of cellulose packing and supramolecular dynamics rather than localized chemical bonds. In brown-rot fungi, the band at 74 cm^{-1} showed a pronounced increase in intensity between two and four weeks, yielding strong positive contributions to the temporal difference spectra (Fig. 13). Increase in intensity of this peak has been correlated to crystallinity loss in cellulose (Agarwal et al., 2018; Agarwal, 2022), thus BR reduces cellulose crystallinity independent of nitrogen. In contrast, white-rot fungi showed no strong positive temporal association with the 74 cm^{-1} feature

under high, moderate, or low nitrogen, instead exhibiting small but consistent decreases over time, potentially reflecting preferential removal of less ordered cellulose regions. Under extremely low nitrogen, however, temporal changes at 74 cm^{-1} in white-rot fungi became positive and approached the magnitude observed in brown-rot fungi. This shift toward crystallinity loss under severe nitrogen limitation may reflect altered enzymatic regulation in white-rot fungi, whose decay strategy relies heavily on extracellular enzymatic systems that are energetically and nutritionally demanding. In this context, increased lattice disruption under extreme nitrogen stress may signal a shift toward a different mode of cellulose modification. The attenuated and condition-dependent response observed in white-rot fungi contrasts with the consistently strong temporal signal in brown-rot fungi and supports a model in which nitrogen availability selectively constrains how white-rot fungi deploy their enzymatic repertoire during cellulose decomposition.

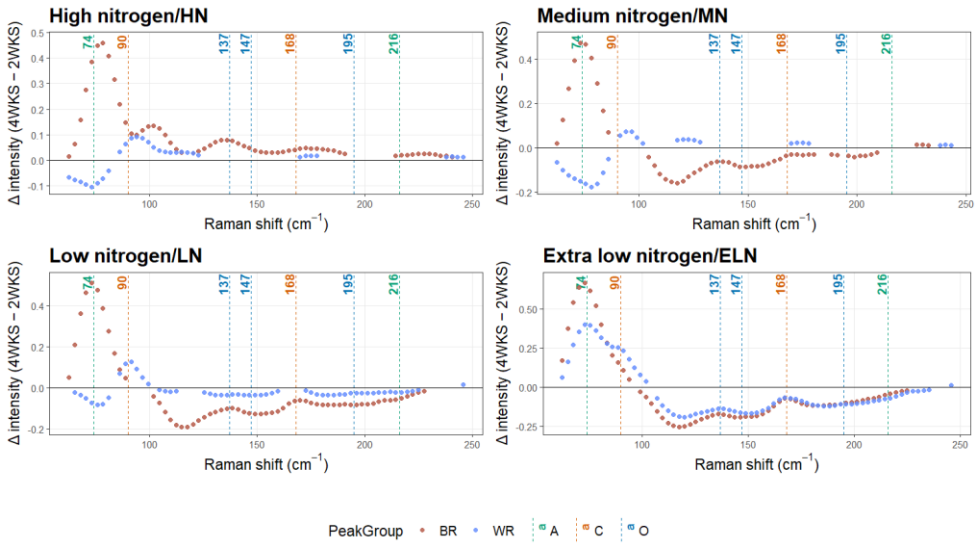

Figure 13. Species-specific time effects on Raman spectra under different nitrogen conditions. For each nitrogen condition (HN, MN, LN, ELN), only wavenumbers showing a significant difference between 2-week and 4-week spectra (FDR-corrected within species) are plotted. The y-axis represents the change in intensity over time (Δ4WKS-2WKS), with positive values indicating increases and negative values indicating decreases. Points are colored by decay type. Vertical dashed lines mark literature-reported wavenumbers, classified into A, C, and O groups and colored accordingly. The solid horizontal line denotes zero change. Together, the panels illustrate how the extent and direction of time-dependent spectral changes vary with nitrogen availability and decay strategy.

Guided by the strong contribution of low-frequency Raman modes, we examined nitrogen and temporal effects at the 74 cm^{-1} feature for each species. Species-level analysis revealed substantial variability within each decay type (Fig. 14). Among brown-rot fungi, temporal changes differed in both magnitude and direction across

species, suggesting that lattice disruption is a shared outcome but is achieved through species-specific trajectories. White-rot fungi likewise exhibited heterogeneous responses, with the strongest temporal effects confined to nitrogen-limited conditions. Together, these results indicate that changes in cellulose lattice dynamics reflect species-level regulation superimposed on broader decay-type patterns.

Figure 14. Direction, strength, and smoothness of intensity change between 2 and 4 weeks for peak at 74cm^{-1} across fungal species, decay types, and nitrogen conditions.
Metrics were calculated as the difference in integrated Raman signal between 4 weeks and 2 weeks (Δ = 4WKS - 2WKS) using 45 replicate measurements per species. Direction indicates whether the net change over time was negative or positive, strength represents the absolute magnitude of this change, and smoothness reflects the variability of replicate responses within each species and nitrogen combination.

Changes at 74 cm^{-1} are therefore best interpreted as reflecting differences in the progression and regulation of cellulose lattice reorganization rather than simple increases or decreases in chemical modification. The prominence of this feature across decay types, combined with its sensitivity to nitrogen availability and time, supports the view that modification of cellulose supramolecular structure is a common target of fungal decay, implemented through distinct regulatory and kinetic trajectories.

Carbon effect is universal, nitrogen effect is decay-specific in saprotrophic fungal transcriptome

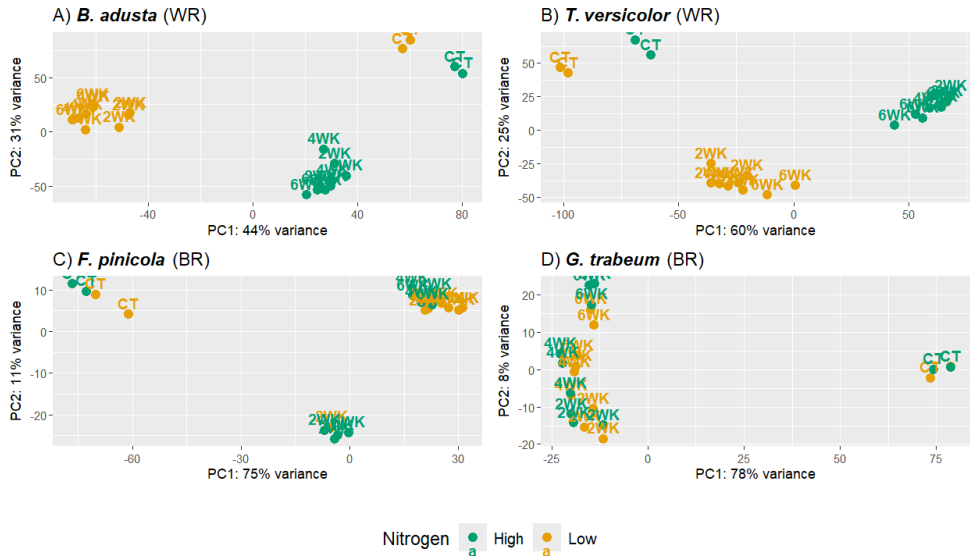

Figure 15.Principal component analysis (PCA) of variance-stabilized counts after performing DESeq2 for four species: *Bjerkandera adusta* (WR), *Trametes versicolor* (WR), *Fomitopsis pinicola* (BR) and *Gloeophyllum trabeum*.

Each point represents a sample. Colors indicate nitrogen treatment (Low and High). Labels indicate the glucose control (CT) and sampling time (2WK, 4WK, and 6WK). Axes represent the first two principal components that harbor the largest source of variance in the dataset

Cotton pellets containing highly crystalline cellulose were colonised by three white-rot fungi (*B. adusta*/BAD, *T. versicolor*/TVE and *P. crispa*/PCR), three brown-rot fungi (*C. puteana*/CPU, *G. trabeum*/GTR and *F. pinicola*/FPI), a litter decomposer (*C. angulatus*/CAN) and a *Stereaceae* isolate (FD-574SV) with an uncertain type of wood decay (GLO) Transcriptomic comparisons revealed a clear asymmetry between carbon- and nitrogen-driven regulation across fungal taxa. Transitioning from glucose to crystalline cellulose elicited a strong and broadly conserved transcriptional response in all fungi examined, independent of decay strategy (Fig. 15). This carbon-driven response involved widespread regulation of genes, underscoring the shared regulatory demands imposed by cellulose recognition and utilization across fungal lifestyles. In contrast, transcriptional responses to nitrogen availability were markedly more variable. While some nitrogen-dependent modulation was observed across species, pronounced regulation was largely restricted to white-rot fungi and CPU (BR), whereas the two brown-rot (GTR, FPI) and wood decayer with uncertain decay type (GLO) showed comparatively

constrained nitrogen responses at the transcriptome level. This divergence indicates that carbon source functions as a primary and conserved driver of fungal gene regulation, whereas nitrogen availability acts as a secondary modulator whose influence depends on decay strategy and ecology. The universality of the carbon response likely reflects the fundamental metabolic reprogramming required to engage crystalline cellulose, regardless of downstream decay mechanisms. In contrast, the variable nitrogen response suggests that nitrogen availability shapes how fungi deploy their decay machinery rather than determining whether it is activated. Stronger nitrogen sensitivity in white-rot and atypical brown-rot fungi may reflect closer dependence between nitrogen limitation and enzymatic modes of substrate modification, while the two brown-rot (GTR, FPI) and wood decayer with uncertain decay type (GLO) may buffer nitrogen effects through non-conventional decay pathways. Together, these patterns support a model in which carbon availability establishes a common transcriptional baseline for cellulose interaction, while nitrogen availability fine-tunes strategy-specific regulatory programs that influence the timing, intensity, and mode of cellulose modification.

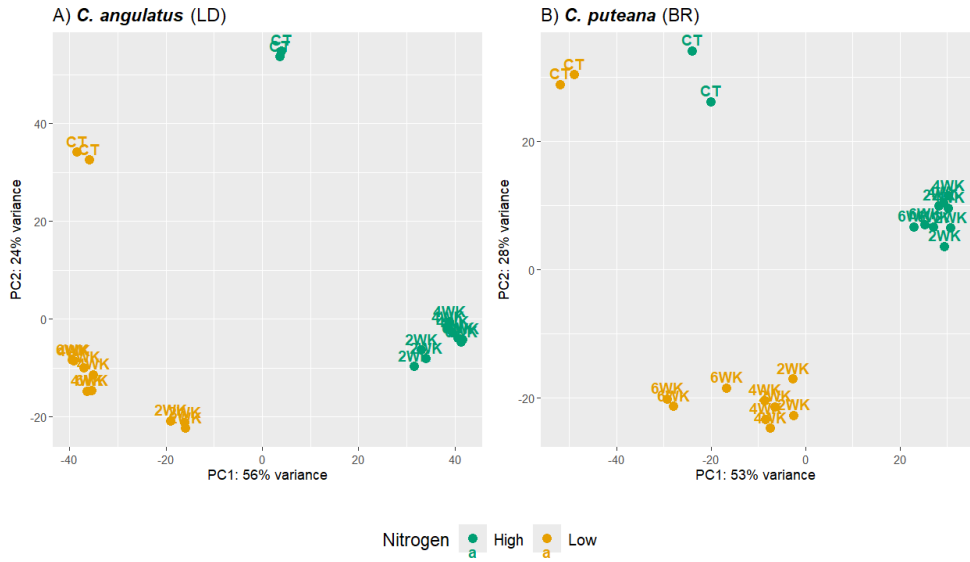

Figure 16.Principal component analysis (PCA) of variance-stabilized counts after performing DESeq2 for two species: *Coprinellus angulatus* (LD), and *Coniophora puteana* (BR).
Each point represents a sample. Colors indicate nitrogen treatment (Low and High). Labels indicate the glucose control (CT) and sampling time (2WK, 4WK, and 6WK). Axes represent the first two principal components that harbor the largest source of variance in the dataset

The brown-rot fungi CPU corresponds with transcriptomic observations of strong nitrogen-responsive regulation (Fig. 16) in contrast to the other two brown-rots (Fig.15). Genomic data puts CPU as atypical along with other brown-rot Boletales

due to its retention of white-rot associated cellulolytic enzymes (e.g., GH6, GH7, increased copy number of AA9) (Eastwood et al., 2011; Floudas et al., 2012; Kohler et al., 2015). Litter decomposers, like WR are also affected by nitrogen availability signalling significant transcriptional regulation. CAN also keeps a high repertoire of enzymes for degrading lignocellulose with AA9 gene copies. (Floudas 2020).

Orthologous genes upregulated during cellulose decomposition by fungi

Although carbon source dominated global transcriptomic organization, decay strategies were associated with distinct expression patterns at the level of functional pathways and orthologous gene groups. These decay-related signatures were not evident as broad transcriptomic separation but became apparent when expression was examined within biologically relevant subsets of genes (Manuscript 2). Pathway-level analyses revealed consistent differences between white-rot and brown-rot fungi in the regulation of carbohydrate-active enzymes, oxidative metabolism, and associated transport functions. White-rot fungi showed coordinated expression of pathways linked to enzymatic cellulose degradation, whereas brown-rot fungi displayed a stronger emphasis on oxidative and redox-associated pathways with more limited induction of hydrolytic systems (Manuscript 2). These patterns were maintained across nitrogen contrasts, indicating that they reflect decay-related regulatory programs rather than nutrient-driven effects. Regulation of some orthologs was conserved (always upregulated) while other orthologs followed a decay type.

Upregulated conserved core of orthogroup categories shared by white-rot and brown-rot fungi

Analysis of orthologs conserved across all six fungi identified a core transcriptional response that was consistently induced during growth on crystalline cellulose relative to glucose, independent of decay strategy. A shared suite of carbohydrate-active enzymes, including endoglucanases (GH12, GH5-5, GH5-22) and β-glucosidases (GH1, GH3), were upregulated at all sampled time points under both high and low-nitrogen conditions (Table 5). These genes have been shown in previous studies to be upregulated in white-rot (Couturier et al., 2015; Kuuskeri et al., 2016; Miyauchi et al., 2020; Hage et al., 2021) and brown-rot fungi (Presley et al., 2018; Zhang et al., 2019; Presley et al., 2020; Figueiredo et al., 2021). The persistence of this response in both white-rot and brown-rot fungi indicates that interaction with crystalline cellulose activates a conserved enzymatic baseline, even among decay strategies that differ substantially in their downstream modes of

substrate modification. This conserved cellulose-induced program was detected separately within each nitrogen condition, demonstrating its robustness to nitrogen availability. Although the core enzymatic composition remained stable, other orthologous groups were upregulated when cellulose utilization occurred under low-nitrogen conditions. These included oxidoreductases, AA3 family enzymes, and expansin-like proteins, suggesting that nutrient context shapes how auxiliary processes are coordinated to facilitate substrate access or offset reduced enzymatic investment, rather than redefining the fundamental response to cellulose. Expansin-related proteins have been shown to be "loosening" agents which increase the accessibility of cellulolytic enzymes (Cosgrove, 2000; Saloheimo et al., 2002; Arantes and Saddler, 2010).

Collectively, these results support a model in which carbon source establishes a shared transcriptional framework for cellulose utilization across decay strategies, while nitrogen contrast modulates the coordination and relative emphasis of this framework. Despite their independent evolutionary histories and distinct decay phenotypes, white-rot and brown-rot fungi therefore converge on a common functional core for cellulose engagement, differing primarily in how this core is integrated with oxidative, structural, and transport-associated processes over time.

Table 5. Conserved upregulated orthogroups categories across three white-rot and three brown-rot fungi grown on cellulose when compared to glucose under high and low nitrogen contrasts. Orthogroup selection was based on their presence across both decay types, their differential expression patterns, and the annotation of the genes within orthogroups. Categories are orthogroups present and consistently upregulated in all six species for all three time points. Background colors: Yellow – CAZYmes, Red – Oxidoreductase activity, Blue – Transporter, Grey – Other. Annotation in bold indicates genes potentially involved in cellulose degradation

High nitrogen contrast	Low nitrogen contrast
CE16	CE16
GH1 β-glucosidase	**GH12 endoglucanase**
GH10	**GH5-5 endoglucanase**
GH12 endoglucanase	**GH5-22 β-xylosidase / endoglucanase**
GH2	**GH1 β-glucosidase**
GH3 β-glucosidase	**GH3 β-glucosidase**
GH3 β-glucosidase	GH5-7 mannanase
GH5-22 β-xylosidase / endoglucanase	**GH3 β-glucosidase**
GH5-5 endoglucanase	**Expansin GH45, endoglucanase-like**
GH5-7	GH2
GH95	D-isomer specific 2-hydroxyacid dehydrogenase, catalytic region
2OG-Fe(II) oxygenase	AA3-3 methanol oxidase
Aldo/keto reductase	Oxidoreductase, N-terminal
Cytochrome P450	Sugar transporter MFS-1
Aldo/keto reductase	MFS-1
Sugar transporter MFS1	Sugar transporter
MFS1	

Decay type dependent upregulation of conserved orthogroup genes

Orthogroup genes that were conserved in all taxa but upregulated in one of the decay types included GH16, and GH18 while for brown-rots, it was β-glucosidase. Expansin-related proteins were upregulated at both nitrogen conditions for BR fungi but only at low nitrogen for WR fungi. WR fungi showed consistent induction of orthogroups linked to polysaccharide remodeling, and cellular or cell-wall-associated processes, including GH16 and GH18 families, and oxidoreductases, together with sustained expression of MFS transporters (Manuscript 2, Table 2 and 3). This pattern suggests that white rot relies on coordinated regulation of enzymatic cellulose processing. In contrast, brown-rot fungi consistently upregulated a broader set of hemicellulose- and matrix-associated glycoside hydrolases, polysaccharide deacetylases, expansin-like proteins, and a wide range of oxidative and transport-related genes (Manuscript 2, Table 2 and 3). This indicates that brown rot emphasizes matrix modification, oxidative chemistry, and transport during cellulose degradation. At low nitrogen contrast, WR fungi produced catalase, usually associated with antioxidant activity (Hansberg et al., 2012). BR fungi, on the other hand, produced a large set of oxidoreductases many of which have been poorly characterized in fungi, and which could be involved in processes related to oxidation of carbohydrates. This indicates that white-rot and brown-rot fungi are distinguished by distinct regulatory programs that control how conserved functions are deployed during cellulose degradation, providing a clear transcriptional basis for the differentiation of decay strategies.

Upregulated decay type specific orthogroup categories

Table 6. Conserved upregulated orthogroups categories across three white-rot but absent in three brown-rot fungi. Comparison was on cellulose when compared to glucose under high and low nitrogen contrasts.

Orthogroup selection was based on their presence in WR but absent in BR, their differential expression patterns, and the annotation of the genes within orthogroups. Categories are orthogroups present and consistently upregulated in all three white-rot fungi for all three time points. Background colors: Yellow – CAZYmes, Red – Oxidoreductase activity, Blue – Transporter, Grey – Other. Annotation in bold indicates genes potentially involved in cellulose degradation

High nitrogen contrast	Low nitrogen contrast
GH6-CBM1	**GH6-CBM1**
AA9	**AA9**
CBM1-GH27	CBM1-GH27
AA9	**AA9**
GH131	**GH131**
CDH	**CDH**
	Cupredoxin
	Sugar transporter, quinate transporter, supressed by sugar

Orthogroups that were both consistently upregulated during cellulose growth and present exclusively in one decay type provide another molecular distinction between white-rot and brown-rot fungi. White-rot fungi uniquely induced a set of cellulose-directed oxidative and hydrolytic functions absent from brown-rot genomes, including AA9 LPMOs, CBM1-linked enzymes (GH6-CBM1 and CBM1-GH27), GH131, and cellobiose dehydrogenase (Table 6). The co-occurrence of AA9, CBM1-containing cellulases, and CDH supports a decay strategy centered on direct binding to crystalline cellulose and coordinated oxidative enhancement of enzymatic hydrolysis, with additional transport functions emerging under the low-nitrogen cellulose versus glucose contrast. The genes detected here have been shown to be present in WR fungi (Floudas et al., 2012; Kohler et al., 2015) but absent in BR fungi (Floudas et al., 2012; Binder et al., 2013; Kohler et al., 2015). In contrast, brown-rot fungi uniquely upregulated orthogroups absent from white-rot genomes that are associated with matrix polysaccharide processing and redox metabolism, including GH43 and GH76, and, under low nitrogen, additional oxidative components such as cytochrome b-245 and aromatic-ring hydroxylase (Table 7). Because these orthogroups are absent from the alternative decay type, these patterns reflect true differences in metabolic capacity rather than regulatory variation, highlighting cellulose surface targeting and LPMO-centered oxidative hydrolysis as defining features of white rot, and matrix-focused modification and redox chemistry possibly representing features of brown rot.

Table 7. Conserved upregulated orthogroups categories across three brown-rot but absent in three white-rot fungi. Comparison was on cellulose when compared to glucose under high and low nitrogen contrasts.

Orthogroup selection was based on their presence in BR but absent in WR, their differential expression patterns, and the annotation of the genes within orthogroups. Categories are orthogroups present and consistently upregulated in all three brown-rot fungi for all three time points. Background colors: Yellow – CAZYmes, Red – Oxidoreductase activity. Annotation in bold indicates genes potentially involved in cellulose degradation

High nitrogen contrast	Low nitrogen contrast
GH43	GH43
GH76	GH76
	Cytochrome b-245, heavy chain
	Aromatic-ring hydroxylase

Special cases of AA9 and GH45 genes involved in decomposition of cellulose by BR and WR fungi

Across decay types, AA9 lytic polysaccharide monooxygenases were consistently upregulated, but their domain architecture clearly differentiated white-rot (WR) and brown-rot (BR) strategies. WR fungi showed the strongest enrichment of AA9 genes and a pronounced association with CBM1 domains, consistent with direct binding to crystalline cellulose and localized oxidative cleavage. In contrast, BR

fungi predominantly upregulated AA9 variants lacking CBM1, suggesting a mode of action less dependent on direct cellulose binding and more closely integrated with broader oxidative processes. Litter-decomposer and unknown wood-decay fungus displayed intermediate patterns, with AA9 induction present in both groups but CBM1-linked forms largely confined to WR and litter decomposer. This distribution suggests that AA9 activity in UWD (GLO) may combine features of both decay strategies, while LD (CAN) shows WR-like characteristics when focusing on AA9 genes. Together, these patterns indicate that CBM1 presence on AA9 enzymes serves as a functional marker distinguishing decay modes. Although AA9 expression in BR fungi has been reported previously (Umezawa et al., 2020; Figueiredo et al., 2021), our data show that this pattern is consistent across lineages, while the functional role of CBM1-lacking AA9s remains unresolved.

Another orthogroup showing contrasting WR and BR expression patterns in relation to nitrogen availability comprises expansin-related proteins (GH45). Expansins have been shown to alter cellulose structure and increase accessibility (Saloheimo et al., 2002; Jäger et al., 2011; Duan et al., 2018), but their role in wood-decaying fungi remains understudied. When integrating transcriptomic and spectroscopic data, it is inherently difficult to directly assign changes in spectral features to specific regulatory shifts in gene expression. Nevertheless, spectroscopic analyses revealed that loss of cellulose crystallinity is a consistent feature of BR decay, whereas in WR fungi it becomes evident only under extremely low nitrogen (ELN) conditions (Fig. 13). Expansins were constitutively upregulated in BR fungi but induced in WR fungi only under ELN conditions, which coincided with spectroscopic signatures of increased loss of crystallinity resembling those observed in BR fungi. This suggests a potential link between expansin expression and amorphogenesis in cellulose in WR under nitrogen limitation, although this relationship requires further experimental validation. From an evolutionary perspective, expansins may represent genes retained from WR ancestors but with altered regulation in BR fungi (Manuscript 2; Tables 2 and 3), contrasting with other cellulolytic genes such as GH6, which appear to have been lost during the transition to the BR decay mode.

Conclusions

Taken together, this thesis demonstrates that fungal cellulose degradation is best understood by integrating chemically informed Raman spectroscopy with transcriptomic analyses. This combined approach reveals how chemical and structural modification of crystalline cellulose is affected by the interaction between enzymatic capacity, regulatory control, nitrogen availability, and time. The main conclusions are summarized below, distinguishing methodological advances from biological insights.

1. Chemically anchored Raman spectroscopy enables detection of biologically meaningful cellulose modification (Manuscript 1)

A key methodological outcome of this thesis is the demonstration that Raman spectroscopy can detect biologically relevant modification of crystalline cellulose when spectral analysis is anchored to chemically defined reference states. By applying controlled oxidation and amorphogenesis treatments, a reduced set of Raman wavenumbers was identified that reflect structural modification of cellulose rather than non-chemical biological variability.

This approach moves beyond reliance on single crystallinity indices or broad spectral regions, showing that chemically informed feature selection improves both interpretation and robustness. Oxidation-associated features were consistently informative, whereas amorphogenesis- and crystallinity-associated features alone showed limited discriminatory power under biological conditions. Importantly, scaling this framework to a larger and more diverse species set revealed that oxidation-based separation weakens as biological heterogeneity increases. Rather than representing a methodological limitation, these results demonstrate that Raman spectroscopy captures chemical gradients rather than enforcing categorical classification, thereby establishing realistic expectations for its application in complex biological systems.

2. Oxidation is a shared but differentially executed axis of cellulose modification (Manuscript 1 – Manuscript 3)

This thesis demonstrates that oxidation is a common component of fungal cellulose modification rather than a defining feature of a single decay strategy. White-rot and brown-rot fungi are therefore not distinguished by the presence or absence of

oxidative mechanisms, but by how oxidative capacity is regulated (AA9), integrated with other enzymatic processes, and expressed at the chemical level.

Oxidative processes give rise to partially overlapping chemical outcomes across taxa, indicating that decay strategies do not converge on discrete oxidation-defined states. Instead, oxidation functions as a unifying and at the same time differentiating axis along which decay strategies diverge through differences in regulatory control and functional integration. This perspective reinforces the idea that oxidation is a shared mechanism that structures variation in cellulose modification.

3. Nitrogen availability modulates decay expression rather than decay identity (Manuscript 2, Manuscript 3)

Nitrogen availability does not define fungal decay strategies but modulates how these strategies are expressed. Across brown-rot, cellulose modifications remained the same under different nitrogen conditions, while transcriptional regulation on cellulose didn't vary with nitrogen limitation. For white-rots, nitrogen regulated their transcriptome while also the modifications on cellulose. The modifications became similar to brown-rot modifications as the nitrogen gets limited.

Nitrogen therefore acts as a contextual regulator that influences the mode of cellulose degradation and redefining decay strategy. This finding underscores the flexibility of white-rot decay systems and highlights the importance of nitrogen in shaping decay expression and identity.

4. Atypical species reveal decay strategies as a continuum shaped by regulation, capacity, and ecology (Manuscript 1 - Manuscript 3)

Evidence from atypical and non-canonical species demonstrates that fungal decay strategies at least regarding cellulose decomposition are better described as positions along a continuum than as discrete functional categories.

Species that deviate from canonical classifications illustrate that neither regulatory responses nor enzymatic repertoires alone are sufficient to define decay strategy. Instead, different decay types occupy stable but overlapping positions along a gradient of decay-related chemical phenotypes. This continuum-based framework takes into consideration differences between transcriptomic and chemical signatures and supports a shift away from binary decay classifications toward a more integrative understanding of fungal cellulose degradation.

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Acknowledgements

Dimitri, how to start this. You're the person everyone goes to for advice, whether it's about fungal research or life in general. When I applied for the position, my confidence was low and it didn't magically appear when I got it either. You helped me build it, step by step. Every time we meet, you ask me the same two questions: how is it going and how are you. And you genuinely mean both. I know the past couple of years haven't been easy for you, especially with Iris being born, but you've kept the same charm when asking these questions just with a bit more dark circles. I'm not someone who expresses things easily, but you've been a great mentor and an even greater friend. Thank you for introducing me to fungi and thank you for believing in me and keeping that faith when I couldn't. "Καθώς προχωρούσα στη διαδρομή αυτή, έμαθα ότι κάποιοι άνθρωποι διαμορφώνουν τον τρόπο που σκεφτόμαστε πολύ πριν το συνειδητοποιήσουμε".

Anders Tunlid, when I started my PhD you were the big-name professor, and I definitely broke a sweat every time I had to explain my work to you. Things got easier once we started working on the first manuscript, it took a long time, but you really helped me improve my writing, my way of thinking, and how to approach complex problems by breaking them down. It's been great working with you on everything from cellulose to chitin. I hope we can keep discussing science now that I know your preferred hangout is Bishop Arms.

Olof, thanks for being such a patient examiner. I know it hasn't always been easy with me playing hopscotch with deadlines. Thanks for all your help and support along the way.

Sara, you've been kind to everyone, even the plants in microbial ecology (though perhaps less so to the glassware). **Mingyue**, you've been a true inspiration when it comes to organising hotpots and for not joining cults. **Sara** and **Mingyue**, Thanks as well for organising so many dinners and events for friends in microbial ecology, even if we never managed to come up with a better name than Party Mycrobes / Funguys. **Lettice**, thank you for being such a thoughtful person, from the Abisko trip to everyday life and for being the only cricket fan. BARMY ARMY! **Albert**, thanks for being the cool kid with two watches and a great football player. **Milda**, thanks for all your help with spectroscopy and interpretation. Good luck in the Netherlands, "So no one told you life was going to be this way." **Hanbang**, you play an instrument, football, tennis, and the group's AI expert. Please stop collecting

hobbies and leave some for the rest of us. **Honorine**, you are a serious cheese and wine enthusiast, and thanks as well for all your help with teaching. **Micaela**, thanks for being you, so cheerful. **Daniel**, thanks for your help during the Abisko trip. **Agnieszka** and **Xiaojing**, thanks for always being so enthusiastic about organising PhD events. **Alice** and **Antonois**, you're microbial scientists at heart, not just aquatic ones, don't let anyone convince you otherwise.

Thanks to **Per Persson** for your input on Raman interpretation. **Emma**, thanks for being an absolute delight and for asking great questions even when you weren't officially the supervisor. **Carl**, amazing work designing OCTAVVS, I hope you're getting more free time now that you're in Medicine. **Calley**, thanks for explaining why we were getting better results with smaller spectral regions; that one was definitely going over my head. **Bowen**, amazing trip to Maastricht with you and **Hanbang**, hope you are having a blast as a professor. **Michiel,** thanks for simplifying complicated spectroscopic outputs.

Sharing an office has been a lot of fun with **Ainara, Carlos** (plays DOTA, but fan of Arcane, weird), and **Sascha** (for the wide-ranging and always interesting conversations, from microbial growth to world politics).

Thanks to **Arne** for organising football over the years, a shame it never quite caught on. And thanks to everyone who joined in over time: **Hanbang, Albert, Sridhar, Qinyang, Gabriel, Stefan**, and many more. Thanks to **Anders Brodin** for introducing me to innebandy, and to **Anders Nilsson** and **Åke** for actually helping improve my game, otherwise I'd still just be running around. Thanks as well to everyone else who played: **Staffan, Gabriel, Yann, Arrian, Jörgen, Erik S, Martin**, and **Mikael**.

Outside of that, thanks to the badminton group, **Joel, Cris, Sara, Margarida, Bowen, Frank L**, and **Julia**. When it wasn't outdoors, it was D&D time. Huge thanks to **Ernö** and **Frank H** for organising, and **to Ara, Marti, Ada, Rebecca**, and **Juan Pablo** for exploring wonderful fantasy worlds together.

Edith, you are one of the nicest people I've ever met, thank you for organising so many events in microbial ecology. Johannes, thanks for making me feel welcome when joining a new group. **Anna S**, thanks for all your help in the lab. **Håkan**, thank you for guiding me through my PhD. **Francois**, you are one of the hardest-working people I know, with an impressively broad knowledge of fungal research. **Mateusz**, thanks for helping me understand Raman data better. **Kristin** and **Ayla**, thanks for the interesting questions about fungal research. **Yiming**, I hope you get better Raman results with your fungi!

Finally, thanks to all the people in microbial ecology over the years: **Anna Goidea, Carla, Manuel, Valentina, Margarida, Iva, Julia, Erik K, Rasa, Cris, Franca, Frank L, Styliani, Fredrik, Vanessa, Katarina, Erika, Roos, Frida, Shillong, Qiuxiang, Xingguo**, and **Yichen**.

Big thanks to the BLAM organising committee, **Sara, Daniel, Katie**, and **Karla**. P.S. it really was the best year for BLAM. Shout-out as well to the BDR committee members: **David, Twinkle, Yun-ting, Robin**, and **Rae**. Special thanks to **Nima Mohseni**, who somehow managed to serve in all the organisations and was always there to help sort out issues for doctoral students.

Ulf Olsson, thanks for the spectroscopy conference. **Henrik Stålbrand**, thanks for being my mid-term opponent, your feedback gave us some really nice ideas on how to move forward. Thanks to **Cheryl** and **Camilla** for accepting me into CLIMBEco. The trips were great, and thanks to all the members for making it such a nice experience. And a huge thank-you to all the administrative staff who keep the building and the department running.

Thanks to **Taina Lundell** for agreeing to be my opponent and for taking the time to read the thesis. Thanks to my committee members **Marie-Noelle Rosso, Sundy Maurice**, and **Heloisa Nunes Bordallo** for being part of the committee and for their time and engagement. And thanks to **Klas Flärdh** for kindly agreeing to step in as a stand-in.

Mirko, thanks for being the first student I supervised. It's a shame I could only keep your interest in fungi and not in spectroscopy.

Mummy, thank you for keeping faith in me when I lost it in myself.

And **Max**, who is a very good boy. Yes, you are. You helped me through a really tough time in my life. 🐾

Fungal cellulose decomposition

Cellulose is the most common organic material on Earth and a key part of plant structure. It gives strength to plant cell walls and is found in wood, leaves, grasses, and everyday materials like paper, cardboard, and cotton. As plants grow, they continuously produce large amounts of cellulose across land ecosystems. However, cellulose is very difficult to break down because its structure is strong and tightly packed, and most organisms cannot use it easily. In nature, fungi play a major role by releasing special enzymes that break cellulose into smaller molecules, which they then absorb as food. By breaking down cellulose, fungi prevent dead plant material from building up in the environment. Without them, fallen leaves and wood would persist for long periods, slowing soil formation and reducing nutrients available for new plant growth. Over time, this could also affect how carbon is stored in the environment. Fungal decomposition is therefore essential for healthy ecosystems, as it helps recycle nutrients, supports soil development, and returns carbon from plants back into the soil and atmosphere. Fungal cellulose breakdown also has practical uses, including composting, waste treatment, and biofuel production. Studying how fungi break down cellulose helps us understand nature and develop more sustainable technologies.

Printed by Media-Tryck, Lund 2026 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

Department of Biology

ISBN 978-91-8104-789-9