THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Discovery and applications of family AA9 lytic polysaccharide monooxygenases

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To Anastasia

Science is not only compatible with spirituality; it is a profound source of spirituality. When we recognize our place in an immensity of light-years and in the passage of ages, when we grasp the intricacy, beauty, and subtlety of life, then that soaring feeling, that sense of elation and humility combined, is surely spiritual.
— Carl Sagan (from “The Demon Haunted World: Science as a Candle in the Dark”)

Preface

This doctoral thesis serves as partial fulfillment of the requirements for obtaining the degree of Doctor of Philosophy at the Department of Life Sciences (previously Department of Biology and Biological Engineering), Chalmers University of Technology, Sweden. The work was performed at the Wallenberg Wood Science Center (WWSC), funded by the Knut and Alice Wallenberg Foundation and Chalmers Foundation. The PhD studies that led to the completion of this thesis were carried out between February 2019 and February 2023 under the supervision of Prof. Lisbeth Olsson and co-supervision of Prof. Vincent G.H. Eijsink and Dr. Olav A. Hegnar. The thesis was examined by Assoc. Prof. Elin Esbjörner.

I performed most of the work presented in this thesis at the Division of Industrial Biotechnology, Chalmers University of Technology. Parts of the HPAEC-PAD and MALDI-TOF MS experiments were carried out during several research visits to the Norwegian University of Life Sciences (NMBU), with grants from the Chalmers Area of Advance in Material Science, under the supervision of Prof. Vincent Eijsink and Drs. Olav Hegnar and Anikó Várnai. MALDI-TOF MS analyses were also conducted at the Chalmers Chemical Imaging Infrastructure.

Monika Tõlgo
January 2023

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Abstract

Auxillary activity family 9 lytic polysaccharide monooxygenases (abbreviated as AA9s or LPMO9s) are fungal mono-copper enzymes capable of oxidatively cleaving various plant cell wall oligo- and/or polysaccharides. LPMO9s are key components of lignocellulolytic enzyme cocktails used in today's biorefineries to break down biomass into fermentable sugars.

Highly stable enzymes with novel functions are of great interest to improve enzymatic biorefinery processes and their economic feasibility. Genome sequencing of an industrially relevant fungus, *Thermothielavioides terrestris* LPH172, revealed 411 putative carbohydrate-active enzyme (CAZy) domains. Transcriptomic analysis indicated that the fungus upregulated numerous LPMO9 genes in concert with canonical cellulase and hemicellulase encoding genes to degrade lignocellulose. Nuanced co-upregulation was detected for LPMO9 genes and those encoding other redox-active CAZymes. Six strongly upregulated *Tt*LPMO9 genes were heterologously expressed and functionally characterized using cellulosic and hemicellulosic substrates. These studies showed that the multitude of LPMO9 genes provided the fungus with different functions, including previously unknown cleavage of cellulose-associated spruce arabinoglucuronoxylan and acetylated birch glucuronoxylan. In a related study, xylanolytic LPMO9 activity was revealed or enhanced by debranching xylans enzymatically, which likely assumed a rigid and stretched xylan conformation that associated with cellulose to increase accessibility to LPMO9s.

LPMOs have unique oxidative powers which render them advantageous for various biorefinery applications. A C1-oxidizing *Tt*LPMO9G was found to increase the amount of carboxyl groups on sulfated cellulose nanocrystals by 10%, without any extensive degradation of the crystals. The functional groups thus generated were used for proof-of-concept crosslinking, which could aid in the production of bio-based materials. In another application, addition of *Ta*LPMO9A to a benchmark LPMO-poor cellulolytic cocktail was shown to improve saccharification yields of mildly pretreated spruce substrates. The final glucose and xylose yields were increased by up to 1.6- and 1.5-fold, respectively, illustrating how LPMO9s can be exploited in the saccharification of these notoriously recalcitrant substrates.

Keywords: thermophilic fungi, carbohydrate active enzymes, LPMO, lytic polysaccharide monooxygenases, AA9, lignocellulolytic enzyme cocktails, lignocellulose, polysaccharides, cellulose, xylan, cellulose nanocrystals, spruce

List of publications

This thesis is based on the five papers listed below. I refer to them in the text by their Roman numerals.

I **Tõlgo, M.,*** Hüttner, S.,* Rugbjerg, P., Thuy, N. T., Thanh, V. N., Larsbrink, J., & Olsson, L. (2021).
Genomic and transcriptomic analysis of the thermophilic lignocellulose-degrading fungus *Thielavia terrestris* LPH172.
Biotechnology for Biofuels, 14(1), 1-16.

II **Tõlgo, M.,** Hegnar, O. A., Østby, H., Várnai, A., Vilaplana, F., Eijsink, V. G. H., & Olsson, L. (2022).
Comparison of six lytic polysaccharide monooxygenases from *Thermothielavioides terrestris* shows that functional variation underlies the multiplicity of LPMO genes in filamentous fungi.
Applied and Environmental Microbiology, 88(6), e00096-22.

III **Tõlgo, M.,** Hegnar, O.A., Larsbrink, J., Vilaplana, F., Eijsink, V. G. H., & Olsson, L. (2023).
Enzymatic debranching is a key determinant of the xylan-degrading activity of family AA9 lytic polysaccharide monooxygenases.
Biotechnology for Biofuels and Bioproducts, 16(2), 1-15.

IV Caputo., F., **Tõlgo, M.,** Naidjonoka, P., Krogh K. B. R. M., Novy, V., & Olsson, L.
Investigating the role of AA9 LPMOs in enzymatic hydrolysis of differentially steam-pretreated spruce.
Submitted for publication.

V Navarro Llacer, S.,* **Tõlgo, M.,*** Olsson, L., & Nypelö, T.
Carboxylation of sulfated cellulose nanocrystals by family AA9 lytic polysaccharide monooxygenases.
Submitted for publication.

*These authors contributed equally.

Contribution summary

I First author (shared). I contributed in part to genomic and transcriptomic analysis. I interpreted the data, put the data in context of fungal lignocellulose degradation and wrote the first draft of the manuscript. I finalized the manuscript in collaboration with all co-authors.

II First author. I conceptualized the study, planned the work, carried out most of the experiments, interpreted most of the data, and wrote the first draft of the manuscript. I finalized the manuscript in collaboration with all co-authors.

III First author. I conceptualized the study, planned the work, carried out the experiments, interpreted most of the data, and wrote the first draft of the manuscript. I finalized the manuscript in collaboration with all co-authors.

IV Second author. I contributed to the conceptualization and planning of the work. I did part of the experiments and analyses, interpreted some of the data, and contributed to the writing of the manuscript. I finalized the manuscript in collaboration with all co-authors.

V First author (shared). I contributed to the conceptualization and planning of the work. I carried out part of the experiments and analyses and interpreted some of the data. I contributed to the writing of the manuscript. I finalized the manuscript in collaboration with all co-authors.

Oral and poster presentations

1. Presentation of the poster “Novel AA9 LPMOs from the thermophilic fungus *Thielavia terrestris* LPH 172” at the Gordon Research Conference “Carbohydrate-Active Enzymes for Glycan Conversions” (New Hampshire, USA, July 2019)

2. Oral presentation “Enzymatic debranching is a key determinant of the xylan-degrading activity of family AA9 lytic polysaccharide monooxygenases” at the 3rd LPMO symposium (Oslo, Norway, 2022)

3. Biannual poster presentations and pitches at the Wallenberg Wood Science Center workshops (Sweden, 2019–2022)

Contents

Abbreviations

- 2,6-DMP – 2,6-dimethoxyphenol
- AA – auxiliary activity
- AA9 – lytic polysaccharide monooxygenase from auxiliary activity family 9 (or auxiliary activity family 9)
- CAZy – carbohydrate active enzyme
- CBM – carbohydrate binding module
- CNC – cellulose nanocrystal
- CNF – cellulose nanofiber
- GH – glycoside hydrolase
- HPAEC-PAD – high performance anion exchange chromatography with pulsed amperometric detection
- LPMO – lytic polysaccharide monooxygenase
- LPMO9 – lytic polysaccharide monooxygenase from auxiliary activity family 9
- MALDI-TOF – matrix-assisted laser desorption ionization – time of flight
- MS – mass spectrometry
- PASC – phosphoric acid swollen cellulose
- STEX – steam explosion
- TEMPO – 2,2,6,6-tetramethylpiperidine-1-oxyl
- TXG – tamarind xyloglucan
- WAX – wheat arabinoxylan

1. Introduction

Humankind owes a large part of its flourishing to the industrial revolution in the 19th century. Unfortunately, the extensive use of fossil resources that followed has substantially altered the climate on our planet, causing severe adverse effects, like extreme weather and loss of biodiversity (1,2). Fossil fuels are also a finite resource: they contain energy that has accumulated over millions of years and some claim that we have already reached or are soon reaching the peak of its use (3). In a broad perspective, my thesis contributes to improving industrial biotechnology processes to generate products that can replace those based on fossil fuels. I have contributed by providing knowledge on **l**ytic **p**olysaccharide **m**ono**o**xygenases (LPMOs) and demonstrated their use in potential industrial biotechnology processes.

In Chapter 1 of this thesis, I introduce the concept of biorefineries and frame my work within the context of wood-based biorefineries. I explain different enzymatic processes used in biorefineries today and how my thesis contributes to the development of some of these. Additionally, I delineate the aims and outline of this thesis.

In Chapter 2, I explain the complex structure of plant cell walls and the related polymers that I have utilized for my thesis work. It is of fundamental importance to understand the chemical composition of plant cell walls and their structural features to understand the functional variability of LPMOs. I also describe various biomass pretreatment methods that are used to make biomass more amenable to subsequent processing. I focus on steam explosion pretreatment as its role in enzyme accessibility was investigated in **Paper IV**.

In Chapter 3, I describe the importance of filamentous fungi as enzyme sources for biorefinery applications. I highlight some of the main differences in fungal biomass degrading mechanisms and introduce the vast array of enzyme classes that microorganisms use for biomass degradation. Furthermore, I introduce the reader to lignocellulolytic enzyme cocktails and their industrial applications. In Chapter 3, I also discuss the results of **Paper I** in which I investigated one specific fungal strain to describe its lignocellulolytic mechanisms. **Paper I** was the basis for my subsequent characterization of new LPMOs belonging to the auxiliary activity family 9 (abbreviated as AA9s or LPMO9s) (**Paper II**).

In Chapter 4, I provide a background on the biochemistry of LPMOs with focus on AA9 family LPMOs as they were specifically investigated and utilized throughout my thesis work. I give an account on the history of LPMO discovery, discuss suggested reaction mechanisms of LPMOs, and present the most important structural features of LPMO9s. I also present what is currently known about the functional variability

and multiplicity of LPMO9s. I thereafter describe my work on the functional characterization of six LPMO9s on a broad selection of substrates (**Paper II**) and outline how these results contribute to the understanding of the functional variability and multiplicity of LPMO9s. Moreover, I discuss current knowledge of the xylanolytic activities of LPMO9s and present how my work in **Paper III** substantially increases the understanding of xylanolytic capabilities of LPMO9s.

In Chapter 5, I focus on potential applications of LPMO9s, particularly for softwood saccharification (**Paper IV**) and functionalization of cellulose nanocrystals (CNCs) (**Paper V**). I describe how LPMO9s are used in lignocellulolytic cocktails today and why they are important for enhancing biomass saccharification. In this context I present the main results of **Paper IV** in which we showed that LPMO9s help to improve the saccharification of mildly pre-treated spruce. I thereafter also describe how LPMO9s have, so far, been used to produce and functionalize bio-based materials, and how my work in **Paper V** brings new possibilities for exploiting LPMO9s for functionalization of CNCs.

Lastly, I conclude the work included in this thesis (Chapter 6) and describe my thoughts on future perspectives regarding the discovery and applications of LPMO9s (Chapter 7).

1.1 Biorefineries

One way to reduce our dependency on fossil fuels and derived products is a shift towards a more bio-based economy, in which different biomasses are utilized as raw materials in biorefineries. Akin to petroleum refineries, biorefineries convert biomass into a spectrum of marketable products, such as fuels, energy, materials, and various chemicals (4). To be economically feasible yet also sustainable, the conversion of biomass needs to be efficient and yield a range of value-added products. As an example, a schematic representation of the processes and products of a Swedish biorefinery, Domsjö Fabriker, is shown in Figure 1. Domsjö Fabriker was established in 1903 as a pulp mill and is currently the largest biorefinery in Sweden (under ownership of the Indian company Aditya Birla). Its main feedstocks are pine and spruce, whereas its main outputs are specialty cellulose, bioethanol, and lignin. These products can be used in clothing, concrete, paints, detergents, energy, and pharmaceutical tablets (5).

Even though the economy prior to the industrial age was also in principle bio-based, the emerging bioeconomy is vastly different as it is driven by science and technology, meaning it is knowledge-based (3). The work presented in my thesis contributes to this knowledge base.

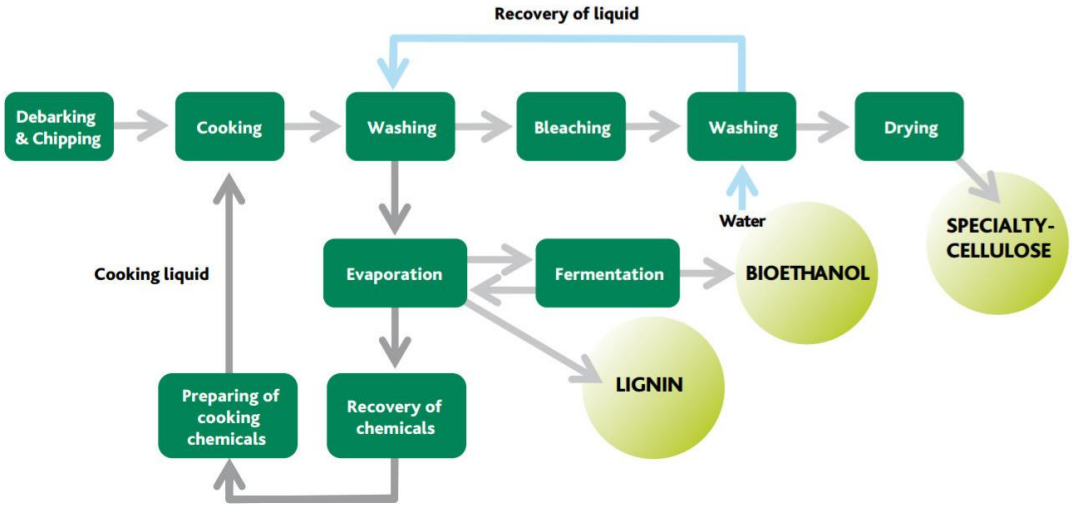


Figure 1. Schematic illustration of the processes and main products of the Swedish biorefinery Domsjö Fabriker. *Retrieved from* (5) *by the approval of Aline Kärrbäck (communications manager for Domsjö Fabriker).*

1.2 Biomass feedstocks

A key requirement for the successful development of biorefineries is the availability of sufficient feedstocks. Often, the choice of biomass is guided by geographic proximity. According to the Food and Agriculture Organization of the United Nations, 11.4 billion tons of dry biomass was available globally in 2011, of which 49% was cellulose/hemicellulose, 23% was sugar/starch, while the rest consisted of protein, oils, fats, and other sources (3). Cellulosic and hemicellulosic materials are the focus of my thesis. Alternative potential feedstocks such as municipal waste are also gaining interest (6). Depending on the source of the feedstock, biorefineries can be classified as forest/wood-based, agricultural, or marine-based. Feedstocks are classified as first-generation when they are derived from food plants (e.g., sugar, starch, oils, and fats) or second-generation, when they are derived from non-food sources such as lignocellulose from wood or the non-edible parts of crops or algae (the latter are sometimes called third-generation feedstock).

The work presented in this thesis focuses mainly on the wood-based biorefinery concept. Approximately 70% of Swedish land is covered with forests (56% with productive forest), of which 40% is spruce (7). Spruce is thus an important feedstock for Swedish biorefineries (and Northern European and American biorefineries), even though spruce and softwood in general are highly recalcitrant to enzymatic hydrolysis (8). The enzymatic degradation of spruce and its constituent polysaccharides are described in **Papers II–IV**, which also address enzymatic conversion of related polysaccharides from other sources.

1.3 Enzymatic processes in biorefineries

Although feedstocks used in biorefineries are of biological origin, not all processes need to be or even can be strictly bio-based. Often, combinations of biological and chemical processes are applied. For example, a sustainable and profitable wood biorefinery system, in which 78% of birch was converted to phenol, propylene, lignin oligomers, and pulp, was recently developed using chemical engineering (9). Nonetheless, according to the principles of green chemistry, the use of toxic (organic) chemicals should be minimized and these compounds should be recycled wherever possible. Use of other harsh conditions such as high temperature should be similarly minimized to reduce the use of energy (10). From both the processing and end-product perspective, there are specific biorefinery steps where enzymes are the most suitable catalysts. They include the release of fermentable sugars from (ligno)cellulosic biomass, and the production of food or pharmaceuticals under strict safety conditions. Enzymatic catalysts are particularly suitable for saccharification, as they have evolved over millions of years to enable bacteria and fungi to maximize energy output from complex biomasses. Additionally, enzymes are generally not toxic and are more biocompatible compared to their chemical alternatives, making them appealing to the food and pharmaceutical industries.

1.3.1 Cellulosic bioethanol

Bioethanol is added to gasoline but can be used also as a green solvent in the pharmaceutical, flavor, and fragrance industries (5). Production of bioethanol in a biorefinery exploits the superb fermentation ability of microorganisms, such as *Saccharomyces cerevisiae* and *Zymomonas mobilis* (4). The European Union aims to derive at least 14% of its transport fuels from renewable sources by 2030, and at least 3.5% should come from advanced biofuels (i.e. second generation biofuels) (11). To achieve this goal, advances in relevant (bio)technologies are required. The prevalent feedstocks for biorefineries today are sugar- and starch-rich crops, which store carbohydrates as monosaccharides and disaccharides, or as easily accessible polysaccharides. Because these sugars can be easily extracted and fermented, their exploitation is cheaper compared to that of more complex biomasses such as lignocellulose (4).

A schematic representation of the steps employed in the production of bioethanol and other biochemicals is shown in Figure 2. Cellulosic bioethanol production encompasses feedstock harvest and logistics, biomass pretreatment, enzymatic saccharification (hydrolysis) of biomass to yield fermentable sugars, fermentation of these sugars to bioethanol, and product formulation (12). Alternative operations of interest include consolidated bioprocessing with or without co-pretreatment (13). **Papers I–IV** of this thesis focus mainly on enzymes that can be applied in the

enzymatic saccharification step in bioethanol production (among other potential applications).

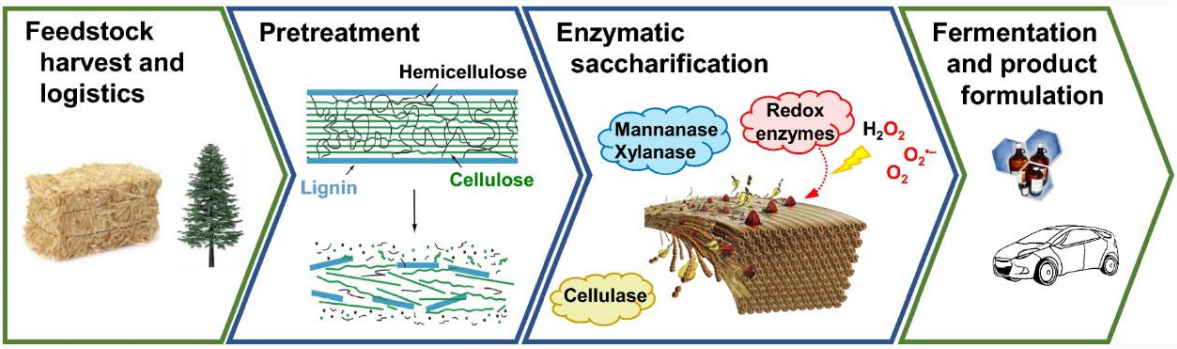


Figure 2. The main processes in bioethanol and biochemical production*. First, the feedstock must be harvested, stored, and transported. Next, the feedstock is pretreated to make different polysaccharides in biomass more accessible for enzymes. This is followed by enzymatic saccharification by different types of enzymes, yielding simple sugars. Last, the sugars are fermented by microorganisms into different products, which generally need down-stream processing and/or product formulation. The figure was retrieved from* (12) *under the CC BY 4.0 license.*

Numerous companies have tried to commercialize cellulosic ethanol, but its production remains a challenge (14), both from an economic (15) as well as a technological point of view (13). One of the main economic impediments is the high cost of enzymes. In 2012, the enzyme cost alone was estimated at 1 USD/gallon of bioethanol produced from poplar (16). At the time, this corresponded to about one-fourth of the cost of a gallon of gasoline in the United States. Hence, lignocellulosic ethanol is hardly an economic alternative to gasoline. While developments in enzyme technology have since then increased the economic sustainability of lignocellulosic ethanol, enzymes remain an important cost factor, especially for highly recalcitrant substrates such as spruce. The main technological issues are non-productive binding of enzymes to lignin, low reaction rates, and enzyme inhibition (17). **Papers I–IV** of this thesis describe the possible use of LPMO9s in enzymatic saccharification of lignocellulose, and how this may bring down the cost of lignocellulolytic enzyme cocktails.

1.3.2 Other possible enzymatic processes

Although the use of enzymes in wood-based biorefineries has so far focused mainly on saccharification, alternative processes have emerged or are under development at proof-of-concept or pilot stages. These include production of cellulose nanofibers

(CNFs), CNCs (see Chapter 5) and other bio-based materials, bleaching of pulp, and production of (prebiotic) oligosaccharides.

Different cellulose-active enzymes have been shown to facilitate the production of CNFs and CNCs, thus providing an alternative to harsh chemicals or energy-demanding mechanical methods (18,19). Additionally, a Swedish university spin-off company, Ecohelix AB has developed an in-line biorefinery process to produce packaging materials from hemicelluloses, whereby the latter are crosslinked by laccases (as stated in their scientific publications) (20,21). Enzymes, such as xylanases and laccases, can be used also for bleaching of pulp, thus replacing traditional toxic chlorine-based reagents (22). Another valorization route involves the selective enzymatic conversion of polysaccharides to oligosaccharides (degree of polymerization 2–10) with potential prebiotic properties (23,24). **Paper V** describes an enzymatic method for modifying sulfated CNCs with LPMO9s, thereby allowing their application in the production of bio-based materials.

1.4 Aims and outline

The general aim of the work described in this thesis was to better understand how fungi degrade biomass in nature, and based on this knowledge, to identify new enzyme functionalities that might improve biotechnological processes, such as biomass saccharification or production of bio-based materials. The specific aim of this thesis was to discover, characterize, and apply a specific class of enzymes – family AA9 LPMOs – in the wood biorefinery context. Unlike cellulases, AA9 LPMOs have been known only for a little over a decade; however, they have already proven to be highly relevant in industrial applications (25–27), and likely new applications will emerge in the future. Yet, a lot remains undiscovered about LPMO9s, for example the full range of their substrate specificity, details of their reaction mechanism(s), structure-function relationships, and optimal reaction conditions, all of which hinder the effective use of LPMOs.

The aims of this thesis were addressed in five papers as outlined below. **Paper I** describes a genome and transcriptome analyses of the industrially important fungus *Thermothielavioides terrestris* LPH172. This fungus had been identified as thermophilic and acidophilic in a screening of Vietnamese fungal strains (28). The main research questions guiding the work in **Paper I** were: *what type of enzymatic machinery does T. terrestris LPH172 harbor to degrade lignocellulose*, and *are there any interesting lignocellulolytic enzyme candidates with possible novel functions for further characterization*? I show that the genome of *T. terrestris* LPH172 encoded more than 400 putative carbohydrate-active enzyme (CAZy) domains. Gene expression analysis furthermore revealed that the fungus transcribed numerous genes encoding LPMO9s, along with genes encoding classical cellulases and hemicellulases when grown on different substrates.

The finding in **Paper I** that *T. terrestris* LPH172 harbors a high number of LPMO9 genes (herein denoted *Tt*LPMO9s to signify the organism from which the LPMO derives) that are upregulated in response to growth on various cellulosic and hemicellulosic substrates led me to investigate the functional variability among these enzymes. The transcriptome analysis in **Paper I** was thus followed up by the heterologous expression and subsequent functional characterization of several upregulated *Tt*LPMO9s (**Paper II**). The guiding research questions in **Paper II** were: *does the high number of LPMO9 genes in T. terrestris LPH172 relate to differences in substrate specificity of TtLPMO9s*, and *can we find novel LPMO9 activities*? Indeed, by functionally characterizing six LPMO9s from this fungus on a broad range of cellulosic and hemicellulosic substrates, including detailed analysis of the reaction products, I demonstrated previously unknown LPMO9 activities on cellulose-associated spruce arabinoglucuronoxylan and acetylated birch glucuronoxylan. Furthermore, the work in **Paper II** corroborated the hypothesis that functional variation determines the high number of LPMO9-encoding genes in filamentous fungi.

Discovery of xylanolytic activity by several *Tt*LPMO9s on chemically different xylans with varied substitution levels (**Paper II**) led me to the next research question: *is the xylanolytic activity of LPMO9s affected by xylan substitutions?* In **Paper III**, I thereafter show that xylan debranching significantly improves the xylan-degrading ability of LPMO9s. In this study I showed that it is possible to boost LPMO9 activity on various xylans, including commercially available xylans with different substitution levels and enzymatically debranched xylans. Moreover, xylan debranching enabled previously unknown xylanolytic activities of one *Tt*LPMO9 (*Tt*LPMO9E) on spruce and wheat xylans. My work suggests that the effects of debranching relate to conformational changes of xylans, which likely made the xylan adsorb onto cellulose and thus more accessible to LPMO9s.

In the study described in **Paper IV**, I evaluated LPMO9s from an applied perspective and showed that LPMO9s aid in improving saccharification of highly recalcitrant spruce substrates. Me and my co-authors sought to answer the three following research questions: *can an LPMO-containing lignocellulolytic cocktail or supplementing a benchmark LPMO-poor cellulolytic cocktail with the well-known Thermoascus aurantiacus TaLPMO9A boost glucose and xylose release from highly recalcitrant mildly pretreated spruce substrates? By which mechanism(s)? Under what reaction conditions?* We found that *Ta*LPMO9A supplementation increased glucose and xylose yields by 1.6- and 1.5-fold, respectively. Additionally, using enzymatic xylan debranching (**Paper III**), we showed that *Ta*LPMO9A cleaved spruce arabinoglucuronoxylan. This was a previously undetected activity by *Ta*LPMO9A and our work may thereby explain a function of this enzyme not described earlier, explaining the increased release of xylose observed when supplementing the benchmark cocktail with *Ta*LPMO9A. In **Paper III**, we also showed that *Ta*LPMO9A activity decreased cellulose crystallinity in some substrates, which likely contributed to the observed increased glucose release.

Lastly, the work in **Paper V** was initiated to further explore the potential of LPMO9s in biorefinery applications. In **Paper V**, I sought to answer the following: *is a cellulose-active TtLPMO9 characterized in **Paper II** (TtLPMO9G) a good biocatalyst for carboxylating (sulfated) CNCs for subsequent crosslinking?* Even though the LPMO released soluble products from both non-sulfated and sulfated CNCs, I was able to detect carboxylic acid functionalities only on the latter. LPMO increased the number of carboxyl groups by 10%, and the LPMO-added carboxyl groups were subsequently used for proof-of-concept crosslinking.

2. Lignocellulosic biomass

In my thesis, I utilized different lignocellulosic biomasses and their components as substrates for growing fungi, as well as for enzymatic reactions. Given that the aim of my research was to describe novel enzymes and their potential applications in wood-based biorefineries, this Chapter focuses on the chemistry and ultrastructure of wood. I describe the intricacy of layered plant cell walls, and why they are of interest for biorefineries, and thus also for my thesis. I also discuss the chemistry of lignocellulosic polymers relevant to my work and the reasons why these polymers are hard to degrade. Lignocellulosic biomass often needs to be processed before enzymatic saccharification to make it easier to degrade – this is called biomass pretreatment. At the end of this chapter, I shortly introduce the steam explosion pretreatment method that was used in **Paper IV**.

All vascular plant cell walls contain lignocellulose – a general term that includes cellulose, hemicellulose, and lignin. The first land plants emerged 475 million years ago (29) and plant cell walls have evolved to support plant growth, as well as to fight off (a)biotic stressors. This explains why the degradation of plant material for industrial purposes is so complex – we are trying to degrade something that nature has been slowly improving for millions of years. Most trees live for 100–200 years (29); hence, they must be particularly sturdy and resistant.

Lignocellulose is the most abundant biopolymer on earth, and the largest reservoir of carbon fixed via photosynthesis (30). Lignocellulosic materials of relevance to biorefineries include herbaceous and woody biomass, such as forestry residues (needles, logging side-products, and bark), agricultural waste (e.g. straws and hulls of wheat, oat, rice, and sugarcane bagasse), wood, energy crops (e.g. miscanthus and sorghum), and municipal waste (31,32). Wood is classified as hardwood (eudicotyledonic angiosperms) or softwood (coniferous gymnosperms). As indicated by the name, the wood of the former is generally harder, although other differences, including fiber length (softwood has longer fibers) and tissue morphology (softwood has a simpler and more uniform structure) exist (33). However, these differences are more relevant for the pulp industry and fall outside the scope of this thesis.

2.1 Layers and ultrastructure of wood cell walls

Lignocellulose in wood, as in higher plants in general, is contained in the cell walls, which are hierarchical structures made of a primary and secondary cell wall (Figure 3). The latter is the thickest and comprises three layers, S1, S2, and S3, of which S2 is the most abundant in both softwood and hardwood (33). Some cells contain another layer inside the secondary cell wall, called the warty layer or tertiary cell

wall, which consists of lignin and hemicelluloses (34). Plant cells are surrounded by a lignin-rich middle lamella that acts as a connecting layer between neighboring cells (33).

Although the exact composition of lignocellulose varies depending on the type of plant, cell, and its developmental stage (30), the main components of wood are cellulose (40%–45 %), hemicellulose (20%–35%), and lignin (20%–30%) (33). The varying lignocellulose composition across species and tissues further complicates the enzymatic degradation of plant cell walls.

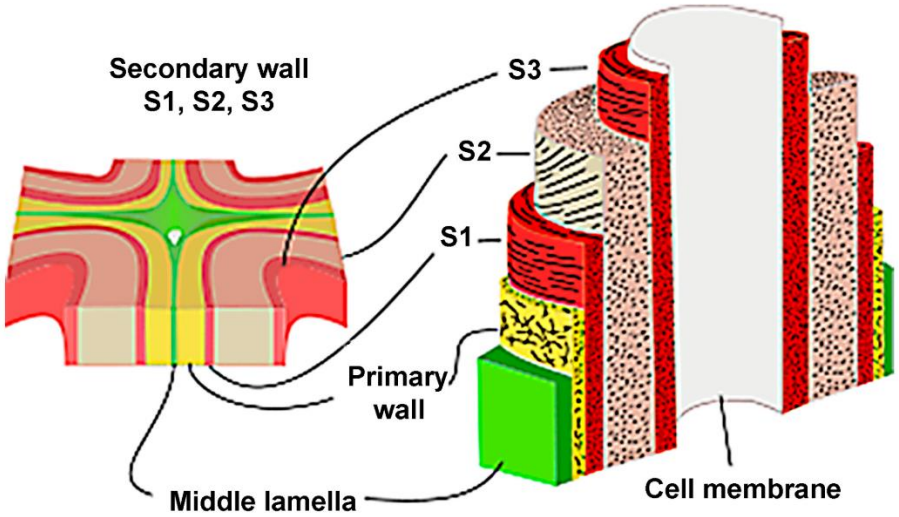


Figure 3. Model of plant cell walls. *The outermost layer, which connects adjacent cells is the middle lamella. The layer immediately below it is the relatively thin primary cell wall, followed by a thicker secondary cell wall (S1–S3 layers) and, finally, the plasma membrane. Note the different angles of cellulose fibrils in the different cell wall layers. The figure was retrieved from* (35) *under the CC BY 4.0 license.*

The primary cell wall is formed during cell division and is relatively thin to accommodate a growing cell (Figure 3). It consists mainly of randomly oriented cellulose microfibrils (33), pectin, and hemicelluloses (36). The secondary cell wall is formed in cells that require rigidity and hydrophobicity to fulfil their transport functions (4,37). Being the thickest layer, it is of interest for biorefinery purposes. The secondary cell wall is composed of cellulose, hemicelluloses, some pectin, and lignin. The latter clearly distinguishes primary cell walls from primary ones (Figure 4). Crucially, the S1, S2, and S3 layers in secondary cell walls have different cellulose fiber orientations (Figure 3), which define the mechanical and physical properties of the fibers (33).

The hierarchical wood structure of secondary cell walls is depicted in Figure 4. Wood is often described as a biocomposite as it is made of multiple biological polymers.

In plant cell walls, cellulose is the “skeletal matrix” which is covered by hemicelluloses and lignin (33). Cellulose is composed of elementary fibrils, sometimes referred to as microfibrils, which according to the current understanding comprise of 18 cellulose chains (38). The elementary fibrils can be assembled into microfibrils with a diameter of 10–20 nm, which can be observed by electron microscopy. The microfibrils can be entangled into larger macrofibrils (33). Depending on the wood species, different hemicelluloses cover the cellulose macrofibrils. Their functions are similar to lignin – to confer structure and protection. A lignin matrix surrounds the hemicellulose-covered cellulose fibrils even further (33). Such a composite structure further complicates plant cell wall degradation (Figure 4).

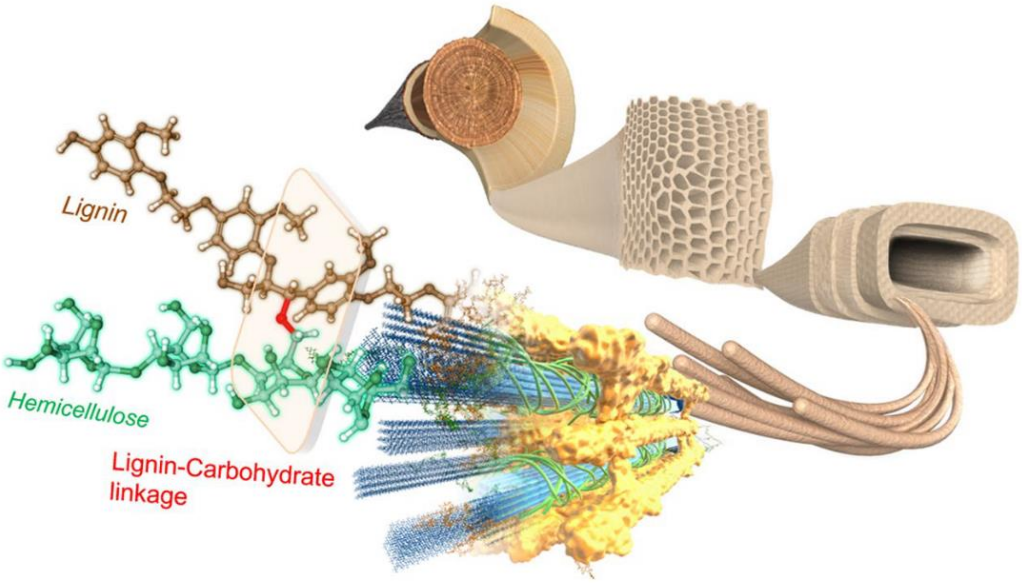


Figure 4. Hierarchical wood structure and ultrastructure of secondary cell walls. *Cellulose is shown in blue, hemicelluloses in green, and lignin in brown. The structure is explained in detail in the main text. The figure was retrieved from* (39) *under the CC BY 4.0 license.*

2.2 Components of lignocellulose

The three main polymers in secondary plant cell walls are characterized by different chemical bonds and properties as presented in the subsequent sections of this Chapter. The diverse bonds in cellulose, hemicellulose and lignin contribute to the complexity and recalcitrance of plant cell walls.

2.2.1 Cellulose

The primary structure of cellulose is a polymer of β-1,4-linked anhydroglucose residues. Because cellulose monomers are positioned at 180 degrees with respect to each other (so-called 2-fold screw conformation), the repeating unit is known as cellobiose (Figure 5) (33). Cellulose and other polysaccharides or oligosaccharides are usually polar, which means they have both a non-reducing and a reducing end (Figure 5). In the former, the terminating sugar is incorporated in a ring; whereas in the latter, it presents a free aldehyde or ketone group that can switch freely from a ring to a chain form (40). The reducing ends are often used to quantify the products of polysaccharide hydrolysis (41,42).

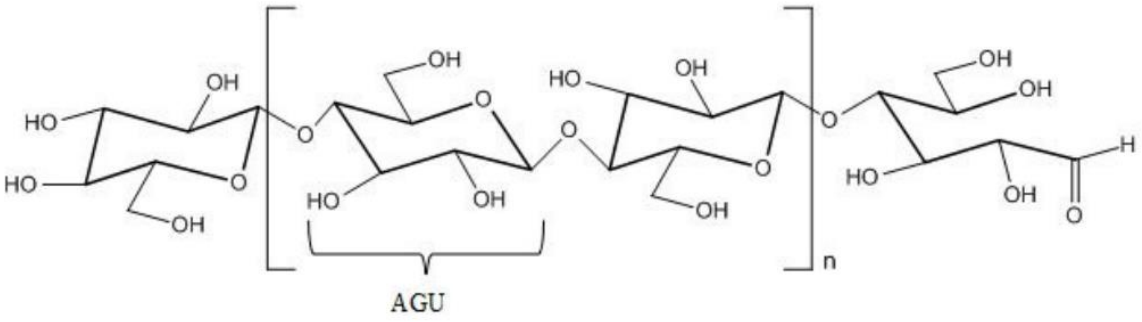


Figure 5. Chemical structure of cellulose indicating the anhydroglucose (AGU) unit, the repeating unit cellobiose (noted in brackets), as well as the reducing end (right) and non-reducing end (left). *The figure was derived from* (43) *under the CC BY 4.0 license.*

Cellulose is synthesized in the plasma membrane by cellulose synthase complexes, from which 18 chains are extruded to generate the 18-chain fibril (38). Cellulose can attain a degree of polymerization of 15 000 monomers, making it one of the longest known biopolymers (33).

It is the secondary structure that makes cellulose an interesting and challenging biopolymer. Intramolecular and intermolecular hydrogen bonds give rise to cellulose sheets, which are held together by van der Waals forces and hydrophobic interactions. Cellulose sheets that bind to each other but do not stack directly on top of each other can form two different crystal forms (allomorphs): cellulose I_α and I_β. Cellulose exists also in polymorphic forms (I–IV) that arise from chemical treatments or are generated naturally in some rare cases. Plant cell walls normally contain the I_β allomorph (33), even though there is evidence that cellulose II hydrate becomes enzymatically saccharified faster than cellulose I_β (44). Cellulose in the secondary plant cell wall is not continuously crystalline but contains also less-ordered regions (also known as amorphous, para- or semi-crystalline cellulose). Notably, cellulose in primary cell walls is less crystalline than in secondary cell walls (45). The tightly packed and solvent-inaccessible crystalline cellulose structure further complicates lignocellulose saccharification.

For decades, researchers have looked for ways to dissolve or modify cellulose to generate unique materials. For example, the highly popular textile material viscose (rayon) is regenerated cellulose and is made either by alkali and carbon disulfide treatment or with *N*-methyl-morpholine-*N*-oxide via the Lyocell process. Carboxymethylcellulose is one of the most important cellulose derivatives, with applications in the food, pharmaceutical, and cosmetic industries. For its preparation, cellulose is treated with strong alkali, after which it is reacted with sodium monochloroacetate to create an ether linkage (33).

In the study described in **Paper II**, I tested celluloses from various sources as substrates for five cellulose-active LPMO9s. Avicel is normally produced from cotton linters and is commonly known as crystalline cellulose, although it has been demonstrated that measurements of crystallinity are complex and different values exist in the literature (46). Phosphoric acid swollen cellulose (PASC) is a common substrate for LPMO analysis and is prepared by mixing Avicel with phosphoric acid. This leads to swelling of the cellulose structure, which becomes more amorphous, while the resulting crystallinity of PASC depends on the exact preparation method used (47).

In the study described in **Paper V**, me and my co-authors investigated the use of an LPMO9 as an alternative to 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) - mediated oxidation, a particularly harsh treatment method. Specifically, in the work shown in **Paper V**, we used CNCs - the crystalline fraction left after treating cellulose fibers with sulfuric acid. CNCs possess charged sulfate half ester groups on their surfaces (Figure 6a and 6b), whose effect on LPMO9 was studied in **Paper V** as will be further presented in Chapter 5. Another common oxidative modification used on CNCs is sodium-periodate oxidation (Figure 6c), described also in Chapter 5.

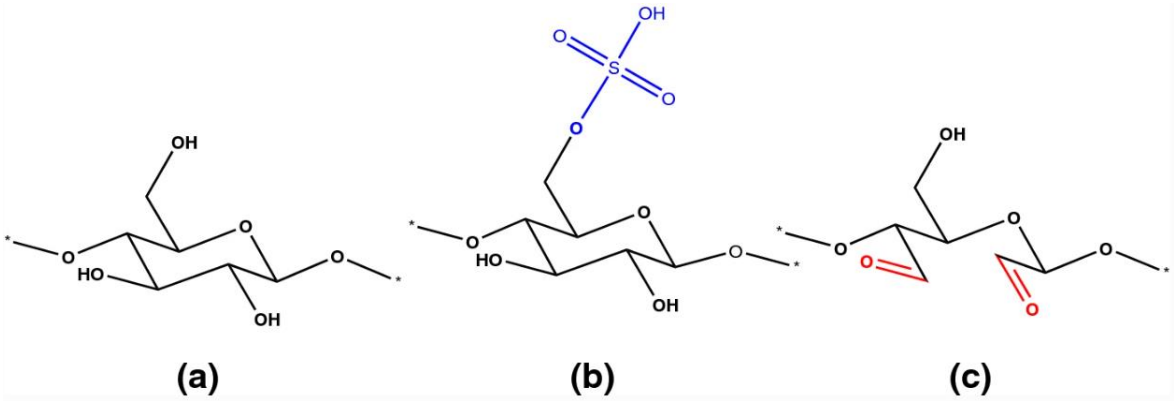


Figure 6. Two types of chemical modifications of CNCs. *(a) Non-modified anhydroglucose. (b) Anhydroglucose containing a sulfate half ester group in the C6-position (blue), generated by sulfuric acid hydrolysis. (c) Anhydroglucose with periodate oxidation at C2 and C3, which introduces aldehyde groups (red). The figure was retrieved from* (48) *under the CC BY 4.0 license.*

2.2.2 Hemicelluloses

Hemicellulose is an archaic term for heterogenous plant cell wall polymers first extracted with alkali in 1891. They were thought to be structurally and chemically similar to cellulose or even cellulose precursors, giving hemicelluloses their ambiguous name (33). According to current understanding, hemicelluloses differ from cellulose, and are divided into subclasses based on the chemical composition of their backbone. Thus, hemicelluloses comprise xylans, (gluco)mannans, xyloglucans, and mixed-linked glucans; although arabinans, galactans, and arabinogalactans are also sometimes classified as hemicelluloses. Unlike cellulose, hemicelluloses are synthesized by glycosyltransferases in the Golgi and are transported to the cell wall via extracellular vesicles (49). Hemicelluloses provide plant cell walls with strength, porosity, and hydration (33). Although still a source of debate in the community, hemicelluloses have been found to bind covalently to lignin, forming so called lignin carbohydrate complexes (Figure 4), which also contribute to the recalcitrance of lignocellulosic biomass. These crosslinks can form either via radical coupling of ferulate moieties on xylan or via coupling of hemicellulosic glycosyl moieties through re-aromatization of lignin intermediates (50).

2.2.2.1 Xyloglucan

Xyloglucan is the dominant hemicellulose in primary cell walls except for grasses and contains a glucan backbone with xylopyranosyl substitutions. The structure of xyloglucan from tamarind seed (TXG), a commercially available substrate, is shown in Figure 7. The TXG structure contains a repetitive motif, whereby three consecutive glycosyl units carry an α-1,6-xylopyranose substitution, while the fourth glucopyranose residue is non-substituted. The xylose decoration can be further substituted with β-1,2-galactopyranose, which in turn can be fucusoylated and acetylated in some species. Less branched xyloglucans are also less soluble (49), but the biochemical role of different substitutions remains to be determined (45). Xyloglucans may assume a flat ribbon-like structure (that can likely adsorb to cellulose) or a twisted conformation, although the exact shape and xyloglucan-cellulose interactions *in planta* and *in vitro* remain to be elucidated (45). TXG mixed with PASC served as a suitable substrate for two *Tt*LPMO9s screened in **Paper II.** The two LPMOs showed distinct cleavage patterns based on substitution sensitivity as will be presented in detail in Chapter 4.

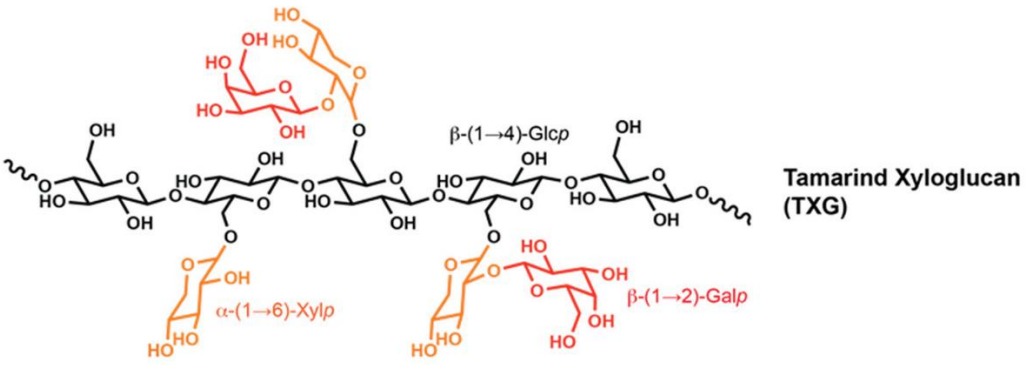


Figure 7. Chemical structure of xyloglucan from tamarind seeds. *The β-1,4-glucopyranosyl (Glc*p*) backbone is shown in black, whereas the α-1,6-linked xylopyranosyl (Xyl*p*) decorations are shown in orange and the β-1,2-galactopyranose (Gal*p*) residues in red. The figure was retrieved from Paper II under the CC BY 4.0 license (and slightly modified).*

2.2.2.2 Xylan

Xylans are the most abundant hemicellulose in secondary hardwood cell walls, where they contribute to 20%–50% of biomass weight, and the second most abundant in softwood, where they account for 5%–15% of cell walls, after glucomannan. Xylans are substituted (also called decorated or branched) polymers, whose backbone consists of xylosyl residues linked via β-1,4-bonds. Depending on the source, xylans can be either highly arabinosylated via single or double α-1,2- or α-1,3-glycosidic bonds (cereals), both arabinosylated via α-1,3-linkages and (methyl)glucuronylated via α-1,2-linkages (grasses, cereals, and softwoods), or both acetylated via *O*-2 or *O*-3 bonds and (methyl)glucuronylated (hardwoods) (Figure 8). Interestingly, the acetyl groups have been shown to migrate along xylooligosaccharides (51). In some cereals, the arabinoxylans can also be feruloylated (49).

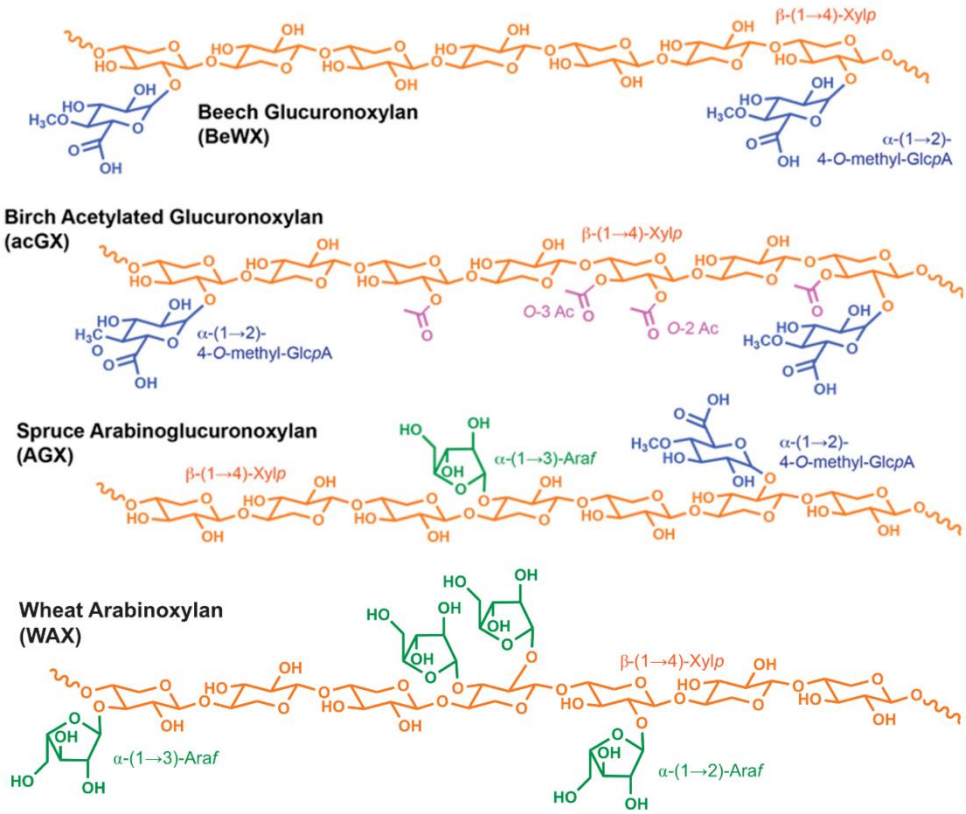


Figure 8. Chemical structure of the different heteroxylans used in this thesis. *The β-1,4-xylopyranosyl (Xyl*p*) backbone is shown in orange, the α-1,2- and α-1,3-arabinofuranosyl decorations in green (Ara*f*), the α-1,2-(methyl)glucuronopyranosyl (4-O-methyl-Glc*p*A) units in blue, and the O-2 and O-3 linked acetyl (Ac) groups in pink. This is a modified and combined figure from Papers II and III (used under the CC BY 4.0 license).*

Some xylans exhibit an even substitution pattern. For example, acetylations in *Arabidopsis thaliana* glucuronoxylan have been demonstrated to be located on every second xylopyranose residue (52,53). Such even substitution pattern is crucial in xylan-cellulose interactions because it promotes the adsorption of xylan as a 2-fold screw onto the hydrophilic surfaces of cellulose. As a result, the unsubstituted side interacts with cellulose via hydrogen bonds (Figure 9). This contrasts with the 3-fold screw conformation assumed by xylan in solutions devoid of cellulose (53–56). Moreover, debranched xylan adsorbs better onto cellulose compared to branched xylan, and the same has been observed with large vs. small xylan molecules (57–59).

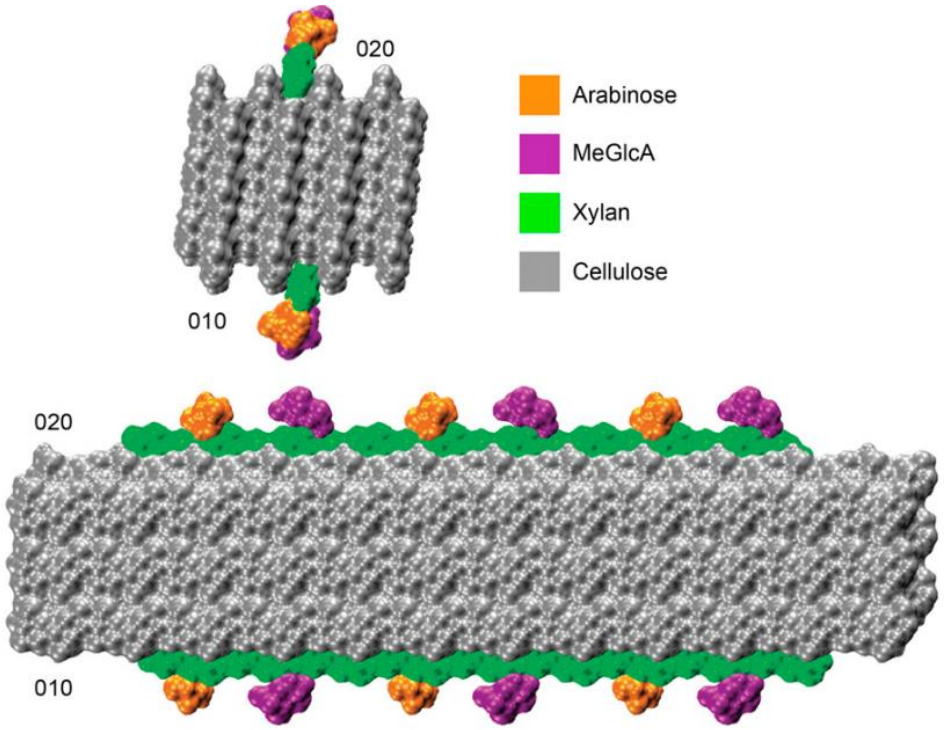


Figure 9. Model of evenly patterned conifer xylan adsorption onto the hydrophilic surfaces (010 and 020) of a 24-chain cellulose microfibril surface. *Reducing end view (top) and side view (bottom) are shown. The figure was retrieved from* (60) *under the CC BY 4.0 license.*

Wheat arabinoxylan (WAX), arabinoglucuronoxylan from spruce, glucuronoxylan from beechwood, and acetylated glucuronoxylan from birchwood were used in **Paper II** to screen for *Tt*LPMO9 activities. Some *Tt*LPMO9s showed xylanolytic activity when the hemicelluloses were combined with PASC (see Chapter 4). Additionally, as assessed in **Paper III** and detailed in Chapter 4, xylan decorations played a key role in the xylanolytic activity of LPMO9s. In **Paper III**, I used commercial arabinoxylans from wheat with different substitution levels, arabinoglucuronoxylan from spruce, and glucuronoxylan from beech as LPMO substrates.

2.2.2.3 Mannan, glucomannan and mixed-linkage glucans

The most abundant hemicelluloses in gymnosperms are galactoglucomannans. Chemical structures of acetylated galactoglucomannan from softwood and acetylated galactomannan from hardwood are shown in Figure 10. Galactoglucomannan consists of a β-1,4-linked non-patterned backbone of glycosyl and mannosyl residues. In softwood, the backbone is decorated with galactosyl moieties via α-1,6-linkages, as well as acetylations at *O*-2 or *O*-3 positions. In hardwood, there are no galactose substitutions. Pure mannans, whose backbone comprises exclusively of mannosyl residues, also exist in nature, as exemplified by

ivory nut mannan. Recent molecular simulation studies suggest that galactoglucomannans in seed mucilage of *Arabidopsis* have a patterned structure and may adsorb to cellulose in plant cell walls (61,62).

In the work described in **Paper II**, extracted acetylated galactoglucomannan from spruce was screened (with or without PASC) as a substrate for *Tt*LPMO9s. However, none of the tested LPMOs showed any activity towards glucomannan or non-branched konjac glucomannan (discussed further in Chapter 4).

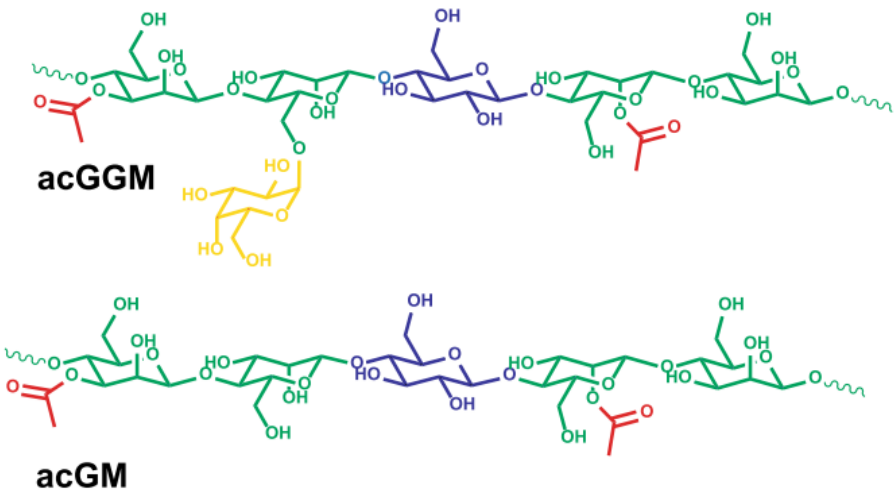


Figure 10. Chemical structures of acetylated galactoglucomannan (acGGM) and acetylated glucomannan (acGM). *The backbone β-1,4-linked glucosyl residues are marked in green and the mannosyl residues in blue. The α-1,6-linked galactosyl substitutions are indicated in yellow and the O-2 and O-3 acetyl groups in red. In Paper II, acGGM from spruce and non-acetylated GM from konjac were used for substrate-specificity screening. The figure was retrieved from* (63) *under CC BY 4.0 license (and slightly modified).*

Another hemicellulose group, called mixed-linked glucans, consists of polymers with cellotriosyl or cellotetrasyl residues interspaced by β-1,3- or β-1,4-linkages (49). These hemicelluloses are found mainly in grasses and were not utilized in the work described in this thesis.

2.2.3 Lignin

Lignin is the second most abundant terrestrial polymer after cellulose. Lignin, a phenolic polymer, reinforces certain types of cell walls, and forms upon covalent binding of its monomers (called monolignols) via radical reactions. The main lignin p-hydroxyphenyl, guaiacyl, and syringyl units are derived from the monolignols p-

coumaroyl (non-methoxylated), coniferyl (monomethoxylated), and sinapyl alcohols (dimethoxylated), respectively (Figure 11).

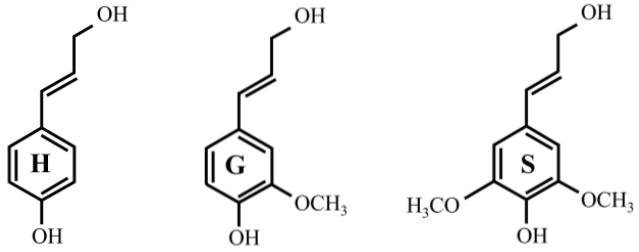


Figure 11. Chemical structures of the main hydroxycinnamyl alcohols (monolignols) that are the building blocks of lignin. *p-Coumaroyl (H unit), coniferyl (G unit), and sinapyl alcohol (S unit) have been retrieved and modified from* (64) *under the CC-BY 4.0 license.*

Monolignols are synthesized in the cytoplasm and then transported to the secondary cell wall, where they are polymerized via phenoloxidase/radical routes. The main bonds in lignin are β-*O*-4 ether linkages, although carbon-carbon bonds have also been detected. Lignin structure and composition are cell type- and species-dependent, as indicated by the absence of dimethoxylated sinapyl units in spruce (64,65). The puzzling structure and characteristics of lignin contribute to the complexity of lignocellulose utilization. The ability of pretreated spruce lignin to act as an electron and/or co-substrate donor to an LPMO9 was tested in **Paper IV** and will be described in more detail in Chapter 5.

2.2.4 Other components

Besides the abovementioned plant cell wall polymers that are of special interest for this thesis, other components include pectin (charged or highly branched polymers of galacturonic acids), extractives (low-molecular mass compounds) such as terpenes and waxes, various proteins, ash (inorganic components), and callose (glucose polymer with β-1,3-linkages) (33). Most of these components are present in relatively small amounts and will not be covered further in this thesis.

2.3 Biomass pretreatment

It is thought that when some fungi start degrading lignocellulosic biomass in nature, they initially use a chemical degradation step to make the biomass structures more accessible to enzymes (66). A parallel can be drawn with biorefineries, where a pretreatment step (Figure 2) allows better access to enzymes used in the subsequent saccharification step. Numerous pretreatment methods have been developed: chemical, physical, biological or a combination of those. Traditional pretreatments include pulping, steam pretreatment (sometimes coupled to explosion), organosolv, and hydrothermal (67). A universal optimal method does not

exist, as the choice of pretreatment depends on the type of biomass and its intended downstream application. If, in the earlier days, the focus was to maximize cellulose accessibility, contemporary biorefineries strive for efficient utilization of all polymeric components (67).

Steam explosion has gathered wide interest, as it can be scaled up and enable mild pretreatment, whereby hemicelluloses are retained and only low levels of microbial fermentation inhibitors (e.g., furfural or hydroxymethylfurfural from hexoses) are generated. At present, this is the most commonly applied pretreatment method, as it requires low capital investments, consumes only moderate amounts of energy, and has a low environmental impact (68).

During steam explosion, water vapor diffuses and condenses in the biomass to heat it up. This process acidifies the liquid phase to pH 3–4, as acids trapped in hemicelluloses become dissolved in solution. Depending on the severity of the method (or if additional acids are added), hemicelluloses become partly or completely hydrolyzed, together with occasional cleavage of lignin ether bonds. At the end of steam pretreatment (usually 5–15 min), the pressure is lowered suddenly, and the evaporation of superheated water occurs. It is this step that causes the biomass to “explode”, rupturing lignocellulosic fibers. In general, the resulting (partial) removal of hemicelluloses and changes to the lignocellulose structure increase enzyme access to cellulose (68).

For the possibility to utilize a mild pretreatment method that retains hemicelluloses, steam explosion was used in the work shown in **Paper IV.** In this paper we exploited three substrates with different levels of hemicellulose retention and studied the effect of LPMO9s on their saccharification (see Chapter 5).

3. Lignocellulolytic enzymes and their discovery

In this Chapter, I explain why fungi are of interest for enzyme discovery and describe the main different mechanisms known today that fungi use to degrade lignocellulose. I present the different enzyme functionalities that are needed for lignocellulose degradation. Additionally, I explain what lignocellulolytic cocktails are and how they are used in biorefineries today. This chapter relates to the enzyme functionalities and substrate preferences that I have worked with throughout my thesis and, more specifically, to the fungal strategy for lignocellulose degradation covered in **Paper I**. As shown in **Paper I** and further discussed in this Chapter, I characterized a novel fungal lignocellulolytic strain *T. terrestris* LPH172 by genomic and transcriptomic analyses to discover possible new LPMO9 functions. These analyses were then the basis for functional characterization and exploitation of novel LPMO9s in **Papers II**, **III** and **V**.

Novel enzymes could facilitate the saccharification of lignocellulosic biomass and open the route for new enzymatic biorefinery applications. Without microbes, cellulose degradation would take millions of years as the *O*-glycosidic bonds are very stable (69). Among the many organisms capable of degrading lignocellulose, including protists, bacteria, oomycetes, and animals (70), filamentous fungi are of the greatest interest for biotechnological applications. First, they are a great source of extremophilic enzymes (in addition to extremophilic bacteria (71)). Second, they can secrete these enzymes in large amounts directly into the extracellular space, unlike some anaerobic bacteria, which employ cell surface-bound multi-enzyme complexes (72). This has sparked profound interest in the mechanisms and enzymes employed by filamentous fungi to deconstruct biomass (73). An example of an industrially relevant fungus, with exceptional lignocellulose-degrading ability is *Trichoderma reesei*, which was discovered during World War II as it degraded the cotton tents and uniforms of the American army (74). *T. reesei* is unique because it possesses only a few cellulase-encoding genes (75) and only three LPMO genes, which are often poorly expressed during growth on lignocellulosic biomasses (76,77). Nonetheless, *T. reesei* remains the main model for fungal lignocellulose degradation and it is known for its high cellulolytic capability (78).

Recent advances in sequencing technologies have enabled the study of genomes (79), transcriptomes, and proteomes/secretomes of fungi (80) (and other microorganisms) at both species and population level through different meta-omic analyses (81,82). The work described in **Paper I** took advantage of this development and scrutinized the genome and transcriptomes of the acidophilic and thermophilic fungus *T. terrestris* LPH172, as will be further described in subchapter 3.6.

Fungi use mainly enzymatic reactions to depolymerize lignocellulose, although a combination of biotic and abiotic reactions has been reported in the case of lignin depolymerization. Most enzymes involved in lignocellulose degradation, except for

some lignin-active enzymes, are included in the CAZy database. The latter uses sequences to group enzymes into six classes, which are further subclassified into families and subfamilies. The six main CAZy classes are glycoside hydrolases, polysaccharide lyases (PLs), carbohydrate esterases (CEs), glycosyl transferases (GTs), auxiliary activity enzymes (AA) and carbohydrate-binding modules (CBMs). The CAZy database encompasses enzymes that degrade, modify or synthesize carbohydrates, along with non-catalytic carbohydrate modules (83,84).

3.1 Fungal mechanisms for lignocellulose degradation

Fungi are among the principal biomass decomposers in nature, using biomass as the main carbon source. Hence, fungi play an essential role in global carbon recycling (73). They do so thanks to their numerous CAZymes, which allow them to degrade polymeric carbohydrates, thus generating monosaccharides or disaccharides for subsequent metabolism. Generally, fungi are classified as white, brown, and soft rot, based on the mechanisms they use to attack lignocellulose and the type of rot they leave behind (73,85,86).

White rot fungi break down lignin to gain access to cellulose in lignocellulosic biomass. Such strategy is employed both by ascomycetes and basidiomycetes. White rot can be further divided into nonselective and selective decay. The former is more typical of hardwood, and implies the simultaneous depolymerization of cellulose, hemicelluloses, and lignin from the lumen towards the middle lamella. During selective decay, instead, lignin and hemicelluloses are attacked before cellulose, and the degradation proceeds from the middle lamella towards the secondary plant cell wall (73,86). Brown rot fungi degrade mainly cellulose and hemicelluloses, and modify lignin only via demethoxylation (73,86). They have more compact and reduced genomes (86), which encode fewer endoglucanases, cellobiohydrolases, and LPMOs (66). Brown rot fungi are mainly basidiomycetes.

Soft rot decay is typical of ascomycetes such as *T. terrestris*, which is characterized in **Paper I.** Soft rot decay can be classified into type I or II. In the former the fungus creates longitudinal cavities within the cell wall, and in the latter the whole secondary cell wall is eroded. Soft rot fungi do not attack the middle lamella and do not employ lignin-degrading enzymes. The major enzymes used are instead cellulases and hemicellulases. This results in extensive loss of carbohydrate polymers which significantly decreases the strength of the wood decayed by soft rotters. The residues of soft rot decay are soft, hence giving this rot type its name (86).

3.2 Cellulose-active enzymes

Cellulose is the most recalcitrant polysaccharide in plant cell walls due to its crystallinity and insolubility in water. According to current understanding, four

classes of enzymes are crucial for synergistic cellulose degradation: endoglucanases, exoglucanases (also known as cellobiohydrolases and CBHs), β-glucosidases (also known as cellobiases), and LPMOs (Figure 12) (85). Given that LPMO(9)s will be discussed in detail in Chapters 4 and 5, I here focus mainly on other cellulose-active enzymes

Cellulose chains in less-ordered cellulose regions are cleaved in the middle by non-processive endo-β-1,4-glucanases (mainly CAZy families GH5, GH7, GH12, and GH45). The resulting open chain ends (either reducing or non-reducing) are points of attack for depolymerization by processive exo-β-1,4-glucanases (CAZy families GH6 and GH7) (Figure 12) (85). Interestingly, cellobiohydrolases are the most abundant enzymes in the secretomes of *T. reesei* (87–89) and are thought to be the main players in commercial cellulolytic enzyme cocktails. They release mainly cellobiose, which is converted to glucose by β-glucosidases (CAZy families GH1 and GH3). The activity of these β-glucosidases is especially important, because cellobiose is an inhibitor of most cellulases (90,91).

The understanding of the enzymatic degradation of cellulose and other recalcitrant polysaccharides became clearer in 2010, when the oxidative mechanism of LPMOs was elucidated by Vaaje-Kolstad and colleagues (92). This discovery opened a new era in the development of lignocellulolytic cocktails. In contrast to cellulases, LPMOs do not use a hydrolytic mechanism but an oxidative mechanism instead. The main role of LPMOs in biomass degradation is thought to be the attack of highly crystalline polysaccharides like cellulose (25). The biochemistry and functions of LPMOs will be discussed in detail in Chapter 4. Of note, fungal secretomes may contain additional redox enzymes that are active on cellulose-derived products, such as cellobiose dehydrogenases (CAZy subfamily AA3_1), cellooligosaccharide dehydrogenases (CAZy family AA7), and glucose oxidases (CAZy family AA3_2), all of which may work in concert with LPMOs (93–96).

Some enzymes depicted in Figure 12 contain additional CBMs. On the one hand, CBMs bring the enzyme closer to the substrate, which benefits enzyme efficiency (97,98). On the other hand, CBMs can also adhere the catalytic domain to the substrate for long periods, stalling the activity. Therefore, the exact role or benefit of CBMs remains unresolved (99,100), and is likely substrate- and enzyme-dependent. Interestingly, in contrast to bacterial polysaccharide-degrading enzymes, fungal enzymes tend to be less modular (101,102).

Not shown in Figure 12 are cell-wall loosening non-catalytic proteins known as expansins, which some plants express during growth (103). Similarly, fungi such as *T. reesei* secrete expansin-like swollenins with analogous function (104). These proteins facilitate enzymatic access to crystalline structures and have in some cases been shown to improve saccharification yields of cellulolytic enzyme cocktails (104).

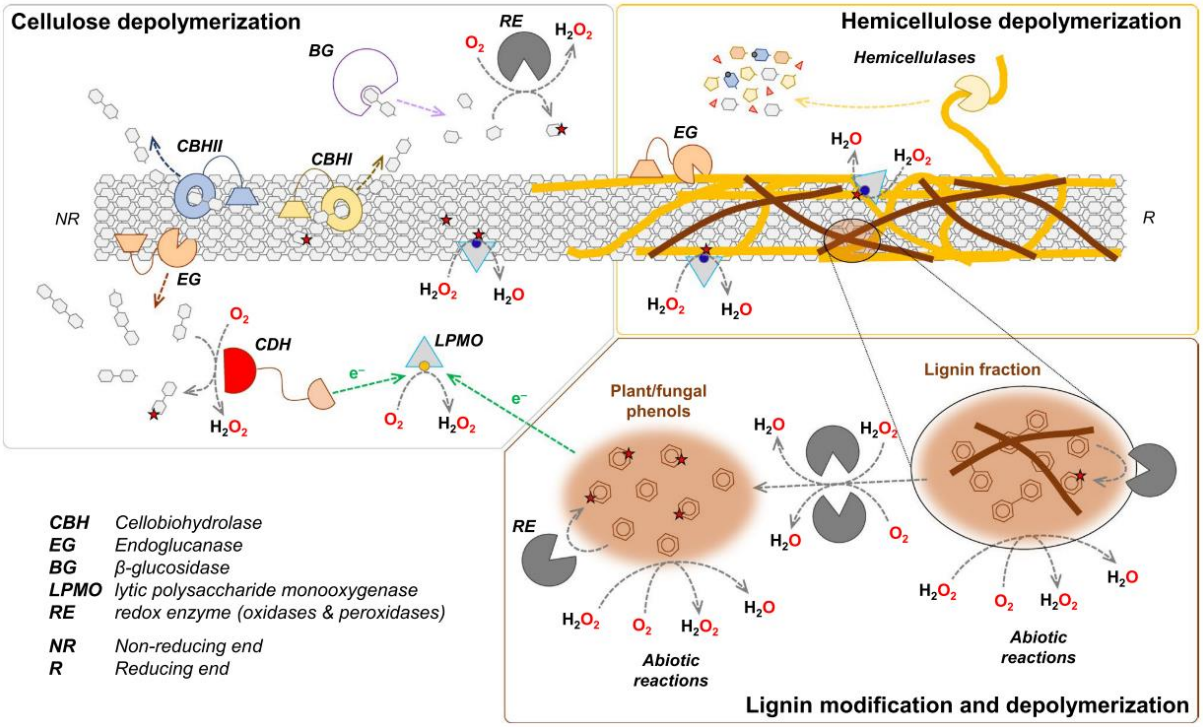


Figure 12. Different abiotic and biotic reactions contributing to the degradation of lignocellulose. *The different (enzymatic) reactions are explained in detail in the main text. The figure was retrieved from* (12) *under the CC BY 4.0 license.*

3.3 Hemicellulose-active enzymes

3.3.1 Xylanases

The degradation of hemicelluloses is not easy as these polysaccharides often contain multiple sugar types, may be branched, and be held together by multiple and varying linkage types. Furthermore, hemicelluloses may form complex co-polymeric structures with cellulose and lignin. The presence of different bonds in hemicelluloses implies the need for multiple cleavage enzymes, even though single enzymes capable of converting xylan to xylose have been described (105).

Depolymerization of the xylan backbone is catalyzed by endo-β-1,4-xylanases, of which the prevalent and best characterized belong to CAZy families GH11 and GH10; although those from families GH5, GH12, GH30, and GH43 have also been shown to possess xylanase activity. According to the current knowledge, GH10s are less sensitive to xylan substitutions than GH11s, but due to their smaller size, GH11s attain better access to more insoluble xylan (106). Xylooligosaccharides released by endoxylanases are further cleaved by β-xylosidases (107). Gool et al. showed that the activity of GH10 on cellulose-xylan complexes was improved by the removal of cellulose, suggesting that some xylanases were not suited to act on xylan complexed with cellulose (108). Other studies have confirmed the inability of xylanases to completely degrade cellulose-bound xylan (109,110).

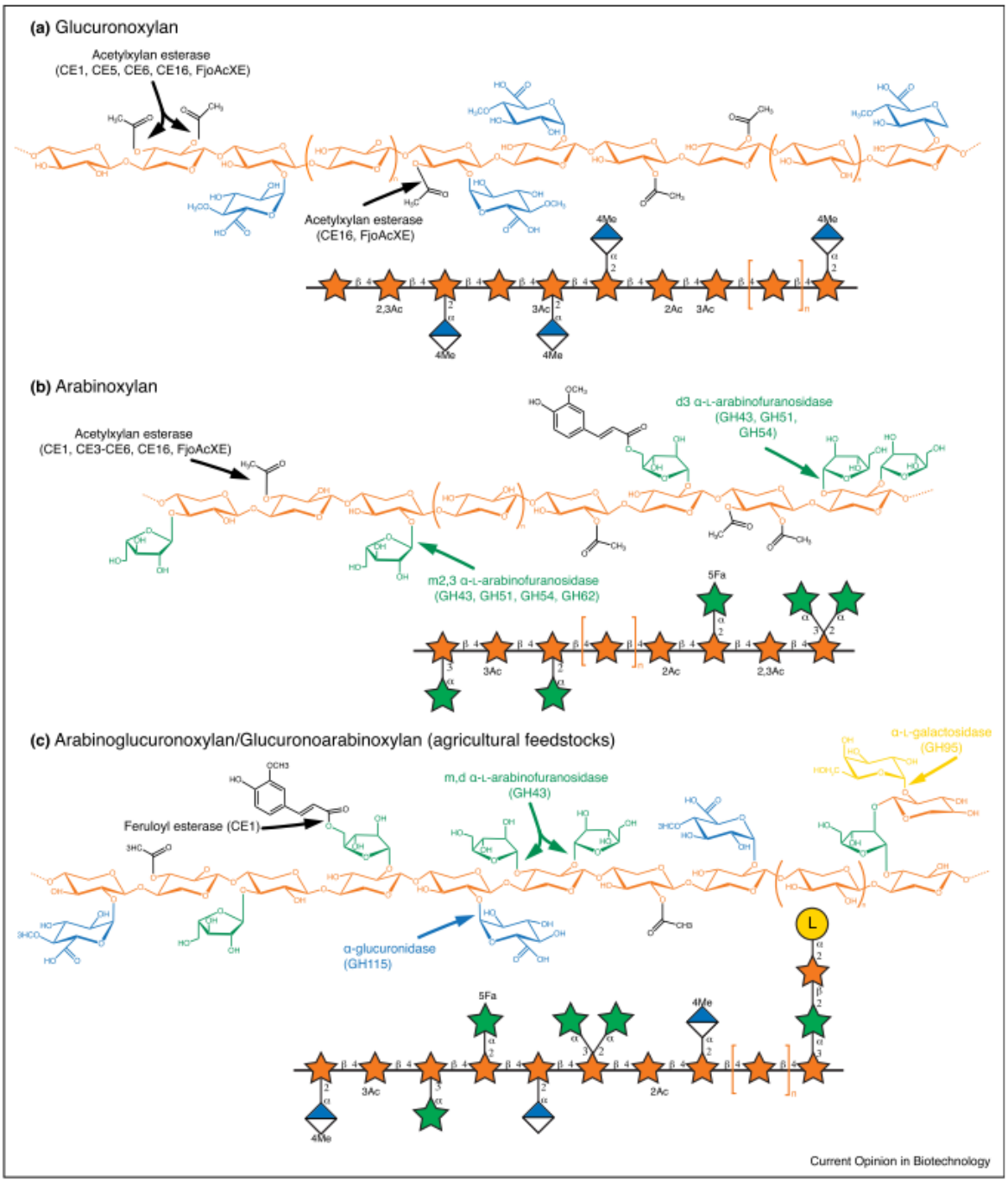


Figure 13. Xylan debranching enzymes. *The enzymes and reactions they catalyse are described in detail in the main text. Ac, acetyl; CE, carbohydrate esterase; Fe, feruroyl; GH, glycoside hydrolase. The figure was retrieved from* (111) *under the CC BY 4.0 license.*

Removal of xylan substitutions requires multiple enzymatic activities, which are summarized in Figure 13. Glycoside hydrolases remove sugars, while carbohydrate esterases (CE1, CE5, CE6, CE16) remove the actetyl and feruloyl groups that decorate some xylans (112,113). Methylated and nonmethylated glucuronic acid groups are cleaved off by GH115s (see **Paper III**). Arabinofuranose substitutions can be cleaved by family GH62 L-arabinofuranosidases, whose activity was

expoited for debranching different heteroxylans in the work described in **Paper III**. Other arabinofuranosidases occur in families GH43, GH51, and GH54; all of them are rather specific for either α-1,2 or α-1,3 branches, but may act on both single- or double-substituted xylose units. Synergy between such debranching enzymes (e.g., GH62s and GH115s) has been demonstrated (114). Finally, glucuronoyl esterases belonging to the CAZy family CE15 are also relevant for xylan degradation, as these enzymes have been shown to cleave lignin carbohydrate complexes between lignin and xylan (115,116).

The role of LPMO9s in hemicellulose (and especially xylan) degradation will be discussed in detail in Chapters 4 and 5, thereby summarizing the results from **Papers II–IV**.

3.3.2 Mannanases and xyloglucanases

To hydrolyze, for instance, acetylated galactoglucomannan in softwood, a combination of different enzyme activities is needed just like for xylans. The key enzymes required for glucomannan depolymerization are endo-β-1,4-mannanases, which release mannooligosaccharides at random from the glucomannan backbone (mainly mannobiose and mannotriose). These enzymes belong to the GH5, GH26, GH113, and GH134 families (117,118). Subsequently, exo-acting β-mannosidases (GH1, GH2, GH5, and GH164) help release mannose as the final product (117,119), both from mannooligosaccharides and mannobiose. Family GH1 and GH3 β-glucosidases are capable of converting glucomannan oligosaccharides to glucose and mannose. Instead, GH4, GH27, and GH36 contain α-galactosidases, which debranch polymeric and oligomeric galactoglucomannan substrates by removing the α-galactoside substituents (117). Acetylations may inhibit glucomannan degradation but can be cleaved by e.g. CE2 family acetyl esterases (120).

The main β-1,4-glucan chain of xyloglucan is cleaved by endo-xyloglucanases from the GH5, GH7, GH12, GH16, GH44, and GH74 families. Of these, GH12 enzymes are the least sensitive to substitutions in the xyloglucan backbone (107).

Some LPMO9s can also act on glucomannan and xyloglucan, as will be introduced and discussed in Chapter 4.

3.4 Lignin-active enzymes

Lignin degradation is accomplished by white rot fungi (and related litter-decomposing fungi) that mineralize lignin to CO_2. This happens enzymatically (i.e., the biotic route in Figure 12) via lignin, manganese, and versatile peroxidases of the AA2 family in the CAZy database, as well as polyphenol oxidases such as laccases from subfamily AA1_1. Brown and soft rot fungi lack genes encoding heme peroxidases, which makes them unable to completely degrade lignin. Accessory

enzymes that can feed H_2O_2 as co-substrate for peroxidases include aryl-alcohol oxidases and glucose oxidases, both of which are classified in the CAZy AA3_2 subfamily.

Enzymatic lignin breakdown may be accompanied by an abiotic radical-based mechanism that uses transition metals such as iron (related to the Fenton reaction), metal-chelators (e.g., oxalate), and radicals generated primarily from H_2O_2. Small hydroxyl and hydroperoxyl radicals can diffuse into the wood more easily than bulkier enzymes, and their destructive action constitutes the first step in both white and brown rot wood degradation processes (66).

Interplay between LPMO9s and lignin-active enzymes has been suggested in several studies. For example, Li et al. showed that the oxidase mechanism of LPMOs was able to drive lignin peroxidase reactions (121). Additionally, Brenelli et al. demonstrated that laccase-derived compounds acted as electron donors for LPMO9s, but laccases and LPMO9s likely competed for the oxygen co-substrate (122). Perna et al. showed that bacterial laccase activity towards lignin generated H_2O_2 which fueled LPMO reactions (123). Finally, Li et al. demonstrated that a combination of an LPMO, hydroquinone, and ferric iron generated hydroxy radicals capable of cleaving lignin carbohydrate complexes or inter-lignin bonds (124). Nevertheless, the exact interaction between LPMOs and lignin-active enzymes, and between lignin and cellulose degradation remains uncertain and further work is needed.

3.5 Lignocellulolytic cocktails

Filamentous fungi are a source of highly stable and abundant lignocellulolytic enzymes. Thus, most commercial (ligno)cellulolytic enzyme cocktails today have fungal origin (80). I refer to lignocellulolytic cocktails as complex mixtures of enzymes, whose different functionalities deconstruct lignocellulosic polysaccharides into fermentable monosaccharides. Often, these enzymes act in synergy. For example, Østby and colleagues have defined synergy as follows: “Synergism between enzymes implies that the concomitant action of the enzymes results in a yield that is higher than the sum of the yields obtained in reactions with the individual enzymes” (12). Some filamentous fungi particularly secrete highly efficient cellulases (85). Enzymatic cocktails consisting of fungal secretomes are used in the industry also for other purposes like food, feed, and textile processing (80). As (ligno)cellulolytic cocktails, for example, *T. reesei* secretomes have attracted interest for their elevated cellulolytic capability, which has been further optimized over time (125). Proprietary *T. reesei* cellulase-containing secretomes are for example marketed and sold as Celluclast by Novozymes. In academia Celluclast is used as a benchmark cellulolytic cocktail with low LPMO activity. Due to its low cellobiase activity, Celluclast is often supplemented with Novozym 188, a β-glucosidase preparation from *Aspergillus niger* (126). Although Novozymes’ enzyme cocktails are most known to the academic community, other biotechnology

companies like Genencor/Dupont also produce them (e.g. Accellerase 1500 from Genencor/Dupont, which is a mixture of *T. reesei* cellobiohydrolases and endoglucanases) (80). Other potential candidates for the production of lignocellulolytic enzyme cocktails are the thermophilic fungi *T. aurantiacus* (94,127) and *Myceliophthora thermophila* (80).

Thermophilic enzymes are of industrial interest for numerous reasons. First, current commercial lignocellulolytic cocktails are most often used at 50°C and pH 5.0. Therefore, all enzymes that could potentially improve these cocktails should be stable and active under the same conditions. In general, thermostable enzymes come with numerous advantages compared to their non-thermostable counterparts: higher stability (allowing for prolonged saccharification times), higher specific activity (allowing for lower enzyme loadings), and their thermostability also increases the flexibility of process set-ups. Of note, enzymes from thermostable organisms do not always maintain thermostability when produced heterologously, as shown by Krska et al. for a multi-domain xylanase-glucuronoyl esterase (128).

Numerous different bonds in several different polymers need to be degraded for the saccharification of lignocellulose (as explained in Chapter 2). Improvements to enzymatic hydrolysis can ameliorate the economic viability of lignocellulosic biorefineries. A great example of the impact of enzyme discovery in this respect is the discovery of LPMOs and the related revelation of their (true, or kinetically more relevant) peroxygenase activity (further discussed in Chapter 4 and 5). The discovery of LPMO9s has marked a new era in the development of lignocellulolytic enzyme cocktails as the novel oxidative mechanism and relatively flat active site of LPMO9s makes them better catalysts to degrade recalcitrant crystalline cellulose. Optimization of the hydrogen-peroxide feeding strategies in biorefineries will likely boost the efficiencies of LPMO-containing enzyme mixtures even further (discussed in Chapter 5). The enzyme discovery covered in **Paper I** led to the characterization of six LPMO9s in **Paper II**. Furthermore, the importance of using the right substrates and auxiliary enzymes for the biochemical characterization of LPMO9s was accentuated by the work described in **Paper III** where previously unknown xylanolytic capabilities were detected for both my new LPMO9s and the well-known *Tt*LPMO9E (**Papers II** and **III**). These capabilities are important as they could further potentiate lignocellulolytic enzyme cocktails.

Lignocellulolytic cocktails can be enhanced via, for example enzyme engineering to improve enzyme kinetics, stability, substrate specificity, while reducing product inhibition and affinity for lignin. These properties represent bottlenecks in existing saccharification processes (129,130). Another possibility for advancement relates to the production of enzymatic cocktails. These enzyme mixtures are used in large amounts, which means that their production should be cheap, and the cocktails should be optimally tailored to the specific substrate. In this respect, on-site production of cocktails that can be fine-tuned to different feedstocks and pretreatments could be particularly beneficial (13,76). Such on-site enzyme production will likely occur in the future, as it is becoming increasingly apparent that lignocellulolytic cocktails need to be substrate- and pretreatment-specific to allow for maximum efficiency and economic feasibility (89,94). Addition of surfactants, use

of immobilized enzymes, and fed-batch saccharification modes can ameliorate process conditions and further lower the costs (17). All in all, there is room to optimize lignocellulolytic cocktails and their use. Unfortunately, the constituents of commercial enzyme cocktails and the steps taken to develop them are not publicly known and it is likely that major cocktail-producing companies, such as Novozymes or Genencor/Dupont, have already implemented critical improvements.

3.6 Identification of the lignocellulolytic machinery of *T. terrestris* LPH 172

In the study described in **Paper I**, I analyzed the newly discovered strain LPH172 (28) of a well-known thermophilic fungus *T. terrestris* using genome and transcriptome analyses. The work aimed to determine which enzymatic mechanisms were employed by this fungal strain to degrade lignocellulosic biomass and its components, and whether novel or interesting enzyme candidates for subsequent functional characterization could be identified. *T. terrestris* strain NRRL 8126 was one of the first two thermophilic fungi, whose genome was completely sequenced (131) and numerous purified enzymes from this fungus had been already characterized previously e. g. in (132,133). In 2011, Berka and colleagues published a general comparison of *T. terrestris* and *M. thermophila* genomes, transcriptomes, and secretomes (131). Prior to **Paper I**, no study had explored the transcriptome of *T. terrestris* growing on various lignocellulosic substrates in high detail.

The newly sequenced genome of *T. terrestris* LPH172 enabled me to screen for putative CAZy domains as indicators of possible lignocellulose-depolymerizing enzymes. The genome of LPH172 contained 10 128 genes with 411 putative CAZy domains. The fungus encoded a relatively high number of putative AA domains (n=83), of which the most abundant were AA7 (n=20), AA9 (n=18), and AA3 (n=16). In principle, the enzymes they encode could all work in synergy, as suggested by the ability of flavoenzymes from both AA3 and AA7 families to promote LPMO9 activity (95,96). With the work presented in **Paper I,** I showed that *T. terrestris* LPH172 encoded a lignocellulolytic machinery theoretically capable of both cellulose and hemicellulose degradation. Gene expression profiles obtained during growth on Avicel, untreated rice straw, and beech glucuronoxylan provided an overview of the genes (and corresponding enzymes) important for the degradation of these substrates by LPH172.

Based on the analysis of highly expressed and upregulated genes on Avicel and rice straw, I concluded that LPH172 expresses a combination of GH5, GH6 and GH7 encoding genes together with LPMO9 genes to degrade cellulose. For xylan degradation, the fungus expressed a combination of GH10 and GH11 genes, different carbohydrate esterase-encoding genes, and some LPMO9 genes (Figure 14). The gene expression analysis corroborated the hypothesis that some of the AA family enzymes worked in concert with LPMO9s as indicated by concomitant upregulation of AA9 genes and two putative AA3 and AA7 family genes. Kracher and colleagues reported that out of 97 tested fungal genomes, 92% encoded LPMOs, but only 58% encoded cellobiose dehydrogenases (subfamily AA3_1);

meaning not all fungi encode AA9s together with cellobiose dehydrogenases (96). Thus, the genome and transcriptome analyses in **Paper I** highlighted that *T. terrestris* LPH172 used a complex and nuanced enzymatic strategy to degrade lignocellulosic biomass and its components.

The transcriptome analysis showed that numerous LPMO9 genes were highly expressed and upregulated on all three substrates, but especially during growth on Avicel and untreated rice straw (Figure 14). According to bioinformatic annotation, *T. terrestris* LPH172 encodes 18 AA9 LPMOs, five AA11 LPMOs, and one AA16 LPMO. The transcriptome analysis showed that among them, 14 family AA9 LPMO genes were highly upregulated. The differential expression of LPMO9 genes on different substrates (**Paper I**) pointed to a varying regulatory mechanism. This could serve as a potential starting point for future investigations on LPMO regulators in filamentous fungi.

As shown in **Paper I**, some filamentous fungi express and use numerous LPMO9s to degrade lignocellulosic biomass. Berka et al. suggested an underlying phylogenetic cause for this as the fungi in the order Sordariales all seemed to possess an extended array of AA9 (then known as GH61) enzymes (131). Further evidence of the key role played by LPMO9s in lignocellulose degradation by *T. terrestris* comes from a study by Merino and Cherry (134), who demonstrated the strong cellulase-enhancing activity of LPMO9s in *T. terrestris* secretomes primed for cellulase production. Hence, the choice made by Novozymes to study *T. terrestris* was likely not accidental, but rational. Notably, **Paper I** also corroborated the synergistic action of LPMO9s with other AA3 family single- and double-domain dehydrogenases, as well as family AA7 oligosaccharide dehydrogenases (95,96).

The number of highly expressed and upregulated LPMO9 genes in LPH172 motivated me to characterize the enzymes they encoded. The differential expression of LPMO9 genes on Avicel, rice straw, and beechwood glucuronoxylan in **Paper I** suggested that LPMO9s had evolved to deconstruct different polysaccharide substrates. The omics-analyses in **Paper I** formed the groundwork for further studies of these highly upregulated *T. terrestris* LPMO9s, as summarized in **Paper II.** Previous evidence on LPMOs had suggested that LPMO9 multiplicity related to functional differences between the enzymes (135–137), but systematic evidence was required to confirm this hypothesis. A summary of LPMO9s selected from **Paper I** and the results of their recombinant production and functional characterization (**Paper II**) is shown in Table 1 (page 45).

Transcript ID	Predicted activity	SP	CAZy-domain(s)	TPM				log2FC			Putative substrate(s)
				Avicel	RS	BX	Glc	Avicel	RS	BX	
TT_00150	Laccase		AA1	6	22	56	7		4	3	L
TT_07374	Multicopper oxidase		AA1_2	9	0	14	3	2		2	L
TT_09261	Laccase	s	AA1_3	3	0	0	0	7	2	3	L
TT_05531	Catalase peroxidase	s	AA2	10	0	62	15			2	
TT_04380	Cellobiose dehydrogenase	s	AA3_1-AA8	1599	4	4	2	9	3		C
TT_08234	Oxygen-dependent choline dehydrogenase		AA3_2	235	2	108	9	5		4	C
TT_03025	FAD-linked oxidoreductase	s	AA7	1	0	0	0	8	5		C
TT_06681	Oxidoreductase	s	AA7	29	8	2	2	4	4		C
TT_02325	Cytochrome-domain containing protein	s	AA8	3	61	0	0	3	10		C
TT_09190	Cytochrome-domain containing protein		AA8	7	30	1	7		5		C
TT_01683	LPMO		AA9	91	6	4	3	5	3		C
TT_01736	LPMO	s	AA9-CBM1	2677	30	20	0	13	9	6	C
TT_03770	LPMO		AA9	41	117	4	10	2	6		C
TT_04350	LPMO	s	AA9-CBM1	4537	9	13	6	10	3	1	C
TT_04352	LPMO	s	AA9	181	234	3	5	5	8		C
TT_05592	LPMO		AA9	11	0	7	1	4	1	3	C
TT_06268	LPMO	s	AA9	7	47	4	0	6	11	5	C
TT_06407	LPMO		AA9	3218	167	32	4	10	8	3	C
TT_07455	LPMO		AA9	8	7	0	0	4	6		C
TT_07456	LPMO		AA9	8862	963	9	4	11	10	1	C
TT_07962	LPMO		AA9	60	14	1	0	10	10	3	C
TT_08370	LPMO	s	AA9	8271	462	21	3	11	10	3	C
TT_09068	LPMO		AA9	0	9	0	0	6	13	3	C
TT_09080	LPMO	s	AA9	1	27	1	1	1	8		C
TT_09453	PQQ-dependent pyranose dehydrogenase		AA12	31	1	120	4	3		5	
TT_05636	Acetylxylan esterase	s	CE1	2	64	0	0	8	16		X
TT_06092	Feruloyl esterase	s	CE1	10	91	6	2	2	8	2	X
TT_02368	Rhamnogalacturonan acetylesterase	s	CE12	2	0	1	0	6	6	4	P
TT_06012	Acetylesterase	s	CE16	915	125	8	8	7	6		X
TT_07717	Multidomain esterase	s	CE3	5	84	1	6		6		X
TT_08970	Multidomain esterase	s	CE3	3	46	1	4		6		X
TT_05762	Acetylxylan esterase	s	CE5	17	16	44	1	5	7	6	X
TT_08166	Acetylxylan esterase	s	CE5-CBM1	3512	84	1329	70	6	3	4	X
TT_08797	Cutinase	s	CE5	2	400	6	8		8		
TT_09441	N-acetylglucosamine 6-phosphate deacetylase		CE9	121	35	1359	66		2	4	
TT_06499	Feruloyl esterase	s	CBM1	6276	40	10	13	9	4		X
TT_04751	Protein phosphatase 1 regulatory subunit		CBM21	278	6	218	48	3		2	
TT_00910	LysM domain-containing protein		CBM50	71	45	1	0	9	11	2	Ch
TT_06197	Endo-β-1,4-xylanase	s	GH10	245	10	1	0	9	7	2	X
TT_08161	Endo-β-1,4-xylanase	s	GH10-CBM1	1335	14	40	25	6	2		X
TT_09033	Endo-β-1,4-xylanase	s	GH10	2	32	2	1	2	8	1	X
TT_01839	Endo-β-1,4-xylanase	s	GH11	1429	8312	419	9	7	12	6	X
TT_02489	Endo-β-1,4-xylanase	s	GH11	11	30	1	1	4	8		X
TT_03075	Endo-β-1,4-xylanase	s	GH11-CBM1	4576	171	299	33	7	5	3	X
TT_03205	Endo-β-1,4-xylanase	s	GH11	4	40	4	0	5	11	5	X
TT_01081	Endoglucanase	s	GH131	666	241	32	32	4	5		C
TT_09709	Feruloyl esterase	s	GH131	416	5	25	13	5	1	1	X
TT_03813	Endo-1,3(4)-β-glucanase	s	GH16	82	4	104	15	3		3	C
TT_08907	β-glucanase	s	GH16	32	23	139	29		2	2	C, XG
TT_04717	Chitinase	s	GH18-CBM18	0	1	13	1		2	4	Ch
TT_05685	Chitinase		GH18	8	17	189	5		4	5	Ch
TT_05010	N,O-diacetylmuramidase	s	GH25	356	249	2452	580		1	2	
TT_06031	Rhamnogalacturonase	s	GH28	201	7	6	2	7	4	2	P
TT_07104	Rhamnogalacturonase	s	GH28	0	1	0	0		5		P
TT_07602	Endopolygalacturonase	s	GH28	2	6	1	0	2	7	2	P
TT_07210	Glycosylceramidase		GH30_7	3659	78	48	7	9	6	3	
TT_02717	Trehalase		GH37	119	4	109	25	2		2	
TT_06379	Endo-α-1,5-L-arabinanase	s	GH43_24	120	1	56	10	4		3	XG, X, P
TT_00222	Arabinan endo-α-1,5-L-arabinosidase	s	GH43_30	4	0	1	0	4		2	XG, X, P
TT_02313	α-L-arabinofuranosidase		GH43_36	53	1	1	2	5			XG, X, P
TT_09000	Endo-β-1,4-glucanase	s	GH45	3188	28	8	96	5			C
TT_00224	Endoglucanase	s	GH5_5	4790	27	7	7	9	4		C
TT_01019	Endo-β-1,4-glucanase	s	GH5_5	2322	26	138	38	6	2	2	C
TT_03238	Endo-β-1,4-glucanase	s	GH5_5	347	1	3	9	5			C
TT_05879	Endo-β-1,4-glucanase	s	GH5_5	31	12	11	1	5	6	3	C
TT_06537	Mannan endo-β-1,4-mannosidase		GH5_7	311	32	3	2	7	7		GM
TT_09640	Mannan endo-β-1,4-mannosidase	s	GH5_7	57	6	1	0	9	8	4	GM
TT_01894	Arabinogalactan endo-β-1,4-galactanase	s	GH53	173	77	5	6	5	6		P
TT_06655	1,4-β-D-glucan cellobiohydrolase	s	GH6	8643	78	90	48	8	3		X
TT_09103	1,4-β-D-glucan cellobiohydrolase	s	GH6	17	3	10	2	3	3	2	C
TT_09005	α-L-arabinofuranosidase	s	GH62	1	13	1	0	4	10	4	XG, X, P
TT_03032	Endoglucanase	s	GH7	170	3	7	4	6	2		C
TT_05512	Endo-β-1,4-glucanase	s	GH7	164	2	0	0	12	8	3	C
TT_05546	Exoglucanase	s	GH7-CBM1	12001	38	26	53	8	2		C
TT_05797	Endoglucanase	s	GH7-CBM1	4654	20	2	20	8	2		C
TT_07042	1,3-β-glucanosyltransferase	s	GH72	7	33	84	5		5	4	
TT_00146	Endo-chitosanase	s	GH75	61	1	19	2	5		3	Ch
TT_03098	Exo-α-L-1,5-arabinanase		GH93	94	23	122	7	4	4	4	P

Figure 14. Top 40 highly upregulated putative CAZyme-encoding genes of T. terrestris LPH172 grown on glucose (Glc), Avicel, rice straw (RS), and beechwood xylan (BX). *TPM, transcripts per million; log2FC, log2 fold change. The putative substrates are abbreviated as follows: C (cellulose), X (xylan), XG (xyloglucan), GM (glucomannan), P (pectin), Ch (chitin), L (lignin). The figure was retrieved from Paper I under the CC BY 4.0 license.*

4. AA9 LPMOs and their functional characterization

Studying the functional variability and different applications of LPMO9s is the core of my thesis work. Consequently, in this Chapter I give a broad background to LPMO9s and LPMOs in general, including their intriguing discovery and suggested reaction mechanisms. Additionally, I cover the oxidative regioselectivity of LPMOs and describe the main structural features of LPMO9s that influence their functional diversity. I also briefly discuss the recombinant production of LPMO9s, as this often poses difficulties. In this Chapter I present the results of **Paper II**, which extends current understanding of LPMO9 multiplicity and functional variability, and of **Paper III** in which I found that the xylanolytic capabilities of LPMO9s are much more prominent than previously known.

4.1 Discovery of AA9 LPMOs

The discovery of LPMOs and AA9s offers an excellent example of the beautiful and often intricate ways natural phenomena may be revealed. The first step towards the discovery of LPMOs occurred in 1950, when Reese and colleagues proposed the C1-Cx theory. The authors sought to determine whether organisms capable of degrading carboxymethylcellulose (they called them non-cellulolytic organisms) could use the Cx factor (i.e., a hydrolytic cellulase) to degrade also native cellulose. Because the Cx factor was not sufficient to degrade native cellulose, the authors hypothesized that true cellulolytic organisms must have another cellulose-active component, designated C1, which initiated the process. Notably, the same study reported that copper and other heavy metals increased the release of reducing sugars (138). In hindsight, this observation suggests that copper-dependent activation of LPMOs in the secretomes promoted the release of sugar from cellulose. Although cellobiohydrolases were initially thought to be the C1 factor, given their catalysis of native cellulose and overall contribution to cellulose saccharification (139), it is accepted today that the C1 role is played by LPMOs (140).

In 1974, a Swedish group published a study, in which they noted that fungal secretomes degraded cellulose more efficiently in the presence of oxygen (141) and that an oxygen-dependent component acts synergistically with cellulases in fungal secretomes. The authors clearly hypothesized the existence of an oxidative cellulase: “We have now discovered yet another enzyme in the culture solution of *S. pulverulentum* which is important for cellulose degradation. The enzyme oxidizes cellulose and its presence during cellulose degradation gives at least twice the extent of degradation given by the same mixture of endo- and exo-glucanases when the oxidizing enzyme is absent.” Nevertheless, LPMOs were not discovered until 36 years later (92), likely because *T. reesei* as the model organism for cellulose degradation does not secrete LPMOs or secretes them only in minute amounts (140).

In 1992, the first gene sequence of what we know is a CBM-containing LPMO from *Agaricus bisporus* was published (142) and the gene product was denoted “Cel1”. Functional characterization of this protein excluded it being an endoglucanase, cellobiohydrolase, xylanase, cellobiose dehydrogenase or β-glucosidase; instead, it demonstrated release of soluble sugars from crystalline cellulose (143). In 1997, Saloheimo and colleagues cloned a “Cel1” from *T. reesei* and classified it as a family GH61 hydrolase, because the enzyme, present in supernatants of GH61-overexpressing yeast strains, showed endo-cellulolytic activity on β-glucan and amorphous cellulose (144). Interestingly, this GH61-encoding gene was co-regulated with other cellulases, suggesting the absence of a GH61-specific regulator in filamentous fungi while confirming the key role of GH61 in cellulose degradation.

In 1998, a virulence factor from the human pathogen *Cryptococcus neoformans* was shown to be closely linked to a Cel1-like gene (145). Today, it is known that this Cel1 is in fact an AA9 family LPMO that contributes to the pathogenicity of *C. neoformans* (146). In 2001, the work on GH61A from *T. reesei* was continued and its functionality was assessed using a purified enzyme (147). In 2003, recombinant production of a GH61 from *Aspergillus kawachii* revealed endoglucanase activity (148), followed by further biochemical and functional analyses (149). Meanwhile, several bacterial “chitin-binding proteins”, originally classified as CBM33s, were reported and are now classified as AA10 family LPMOs (150,151). In two crucial 2005 papers, the crystal structure and functional role of a chitin-binding protein from the chitin-degrading bacterium *Serratia marcescens* were elucidated (152,153). This protein, called CBP21, boosted the activity of traditional chitinases when degrading crystalline chitin. Moreover, the protein presented highly conserved and solvent-exposed hydrophilic residues, whose mutation impaired binding to chitin and failed to promote chitinase activity (152,153).

Secretomes of *T. terrestris* were highly relevant for the discovery of LPMOs as well, as briefly explained in subchapter 3.6. Merino and Cherry from Novozymes reported in 2007 that *T. terrestris* secretomes boosted Celluclast activity on acid-pretreated corn stover. The *T. terrestris* secretomes were further fractionated to detect the contributing enzymes, which identified GH61s as the main synergistic contributors. The investigations concluded that GH61 inclusion of less than 5% of total protein loading enabled a two-fold reduction in total cellulase loading (134), though not on model cellulosic or hemicellulosic substrates. This observation suggested that another plant cell wall component (lignin or hemicellulose) was required to boost cellulase activity. In 2010, Novozymes was granted a patent for “polypeptides having cellulolytic enhancing activity and polynucleotides encoding same” (today known as AA9 LPMOs). Interestingly, the patent was filed already in 2005 (154). In 2008, Karkehabadi and colleagues solved the first crystal structure of a GH61 from *T. reesei*, noting the absence of a catalytic or binding cleft typical of glycoside hydrolases, but strong structural resemblance to CBP21. They also noted conserved polar residues on the surface of the enzyme, and demonstrated that it bound a metal in a site containing two conserved histidines (155).

The real breakthrough came in 2010, when Vaaje-Kolstad and colleagues demonstrated that CBM33 cleaved chitin via an oxidative mechanism, although the

details of the mechanism remained puzzling. They also showed the importance of divalent metal ions in catalysis and revealed the dependence of these novel enzymes on reductants such as ascorbic acid. The authors hypothesized that similar enzymes likely cleaved crystalline cellulose, too (92).

In 2010, Harris et al. studied GH61E from *T. terrestris* (now known as *Tt*LPMO9E) and GH61A from *T. aurantiacus* (now known as *Ta*LPMO9A) (156). The crystal structures of *Tt*LPMO9E and *T. reesei* LPMO9B helped elucidate the metal-binding histidine brace in the enzyme's active site. The nature of the chemical reaction and which metal was involved in promoting cellulase activity remained nevertheless unknown. One year later, studies by Quinlan et al., and Philips et al. showed that GH61 enzymes are also oxidative enzymes and work in a similar manner to CBP21, but on cellulose instead. They were also able to demonstrate that GH61s are mono-copper enzymes with a conserved histidine brace (157,158). In 2011, *Phanerochaete chrysosporium Pc*GH61D was shown to be a cellulose-active metalloenzyme by Westereng and colleagues (159), while Forsberg et al. described the first CBM33 acting on cellulose (160). After that, research on LPMOs truly took off, focusing on the characterization of LPMOs from various organisms, mechanistic studies, implementation of LPMOs in biomass saccharification, and assessment of the role of LPMOs in different pathogenic functions. Since 2013, LPMOs are classified into eight AA families in the CAZy database: AA9–AA11 and AA13–AA17 (83,84). It is likely that more LPMO or LPMO-like families will be identified in the future, as new omics-methods allow for a more direct and rational discovery compared to traditional biochemical analysis of microbial secretomes.

Family AA10 LPMOs are found in bacteria, archaea, viruses, and even fungi, but only bacterial AA10s have been characterized in-depth so far (161). AA10s are active mainly on chitin and cellulose (162). An AA10 LPMO has been suggested to cleave pure beech glucuronoxylan, although evidence is limited (163), while another has been reported in ferns (164). So far, only a few members of the AA11 family of fungal LPMOs have been characterized, and all are active on chitin and/or chito-oligosaccharides (165–168). Family AA13 LPMOs also derive from fungi, and currently only two have demonstrated activity on starch (169,170). Given the existence of starch-degrading amylose hydrolases, it is unclear why starch-active LPMOs would have evolved, as amylose is not crystalline. AA14 LPMOs were discovered in fungi in 2018 and were initially shown to be active on cellulose-associated xylan (171,172). However, Jean-Guy Berrin, the last author on the paper reporting the initial discovery of AA14s disclosed in November 2022 at the 3rd LPMO symposium that his team had been unable to reproduce the xylanolytic-promoting activity of AA14s. Hence, the true AA14 functionality remains unknown. Family AA15 LPMOs are found in viruses and eukaryotes, and five have been characterized so far, with reported activity on cellulose and chitin (173–175). Only a single AA16 (fungal) has been characterized and confirmed to degrade cellulose (176). In 2021, Sabbadin et al. described an AA17 from an oomycete potato pathogen, which was shown to be active on pectin (174). Additionally, there are non-catalytic proteins called X325, which share an LPMO fold and bind copper, but have no catalytic activity on polysaccharides, although they might be involved in pathogenicity (177). Recent studies have focused on the role of LPMOs in

pathogens since several studies have shown that LPMOs are virulence factors. For instance, the pectinolytic AA17 LPMO described above in this paragraph was shown to be a virulence factor in oomycetous plant pathogens (174), while another study showed that an AA10 family LPMO is crucial for infectivity by the human bacterial pathogen *Pseudomonas aeruginosa* (178). Interestingly, at the time of finalizing this thesis, a study reporting *T. terrestris* as a plant pathogen was just published (179). Thus, further studies are required to elucidate if LPMO9s also contribute to the suggested pathogenicity of *T. terrestris*.

4.2 LPMO mechanism and regioselectivity

Currently, two reaction mechanisms are still suggested for LPMOs, a monooxygenase and a peroxygenase reaction (A and B in Figure 15, respectively). However, recent studies point to the latter only: H_2O_2 is likely a more suitable co-substrate as it gives orders of magnitude faster reaction kinetics (166,167,180–186), and H_2O_2 feeding can significantly improve industrial saccharification yields by LPMO-containing cocktails if supplemented in a controlled manner (explained further in subchapter 5.2.1) (187,188). Using an H_2O_2 microsensor in a physiologically relevant environment, Chang and colleagues demonstrated that, when acting on native poplar wood cell walls, LPMO9s consumed only the H_2O_2 generated by cellobiose dehydrogenases, while no O_2 consumption was detected (189). Nonetheless, LPMOs remain classified as oxidases EC 1.14.99.54 (C1-specific dehydrogenation on cellulose), EC 1.14.99.56 (C4-specific dehydrogenation on cellulose), EC 1.14.99.53 (chitin), and EC 1.14.99.55 (starch) (190).

LPMOs potentially engage in multiple productive and non-productive reactions (Figure 15). The initial step in both monooxygenase and peroxygenase reactions is the reduction of Cu(II) in the active site to Cu(I), also called priming reduction (reaction 0 in Figure 15). Suitable electron donors for LPMOs vary from small molecules, such as ascorbic acid, gallic acid, and L-cysteine (56), to lignin-derived compounds (122,191–195) and even other enzymes, such as cellobiose dehydrogenases, oligosaccharide dehydrogenases, and single-domain flavoenzymes, all described in subchapter 3.6 in relation to **Paper I** (95,96,196). Light-induced photosynthetic pigments have been shown to reduce LPMOs and generate H_2O_2 (197–199). Interestingly, reduced LPMO9s seem to bind better to their polysaccharide substrates (200,201), suggesting that active enzymes occur primarily in the substrate-bound state, thereby preventing non-productive reactions.

The choice of reductant is crucial as it affects reaction stability of LPMOs, because it drives *in situ* generation of H_2O_2, which may eventually damage the enzyme (181). Moreover, addition of the reductant should not increase the cost of enzymatic saccharification. Notably, transition metals such as copper affect *in situ* production of H_2O_2 and complicate reaction control. Gallic acid does not display this copper sensitivity and generally ensures stable reactions (202), and was used as a reducing agent in **Papers II** and **III**. Ascorbic acid has been shown as a good reducing agent in more applied conditions, and was thus used in the works described in **Papers IV**

and V. As mentioned above, native lignin or its components may also provide the necessary reducing equivalents (**Paper IV**).

In the peroxygenase reaction, Cu remains in a reduced state over multiple catalytic cycles (reaction 1 in Figure 15). Only H_2O_2 is needed as a co-substrate to deliver the required protons and electrons (181). In the case of the monooxygenase reaction, catalysis requires the acquisition of two protons and an electron, although the source remains unknown. In both the monooxygenase and peroxygenase mechanisms, Cu(I) reacts with the respective oxygen co-substrate, creating a reactive radical intermediate, the nature of which is still to be fully elucidated. This radical abstracts a hydrogen atom from the polysaccharide, followed by a so-called oxygen rebound that leads to a hydroxylation that destabilizes the glycosidic bond, leading to chain cleavage (158,203,204). This reaction results in either C1 (92,160,205) or C4 oxidation (206,207), although some LPMOs generate mixtures of C1- and C4-oxidized products and, hence, also double C1/C4 oxidized products can occur (157,208) (Figure 16). A recent study by Sun et al. suggests that double C4/C6 oxidation by LPMOs can also take place (209). In fact, even C1/C6 double or triple oxidations have been proposed (210,211), but it remains unclear if such products are biologically relevant or they arise solely from over-oxidation. In any case, it has become clear that oxidative regioselectivity depends not only on the LPMO but also on the substrate, as shown e.g. in **Paper II** and in another study by Sun et al. (212). The most common products obtained in LPMO reactions with cellulose are shown in Figure 16.

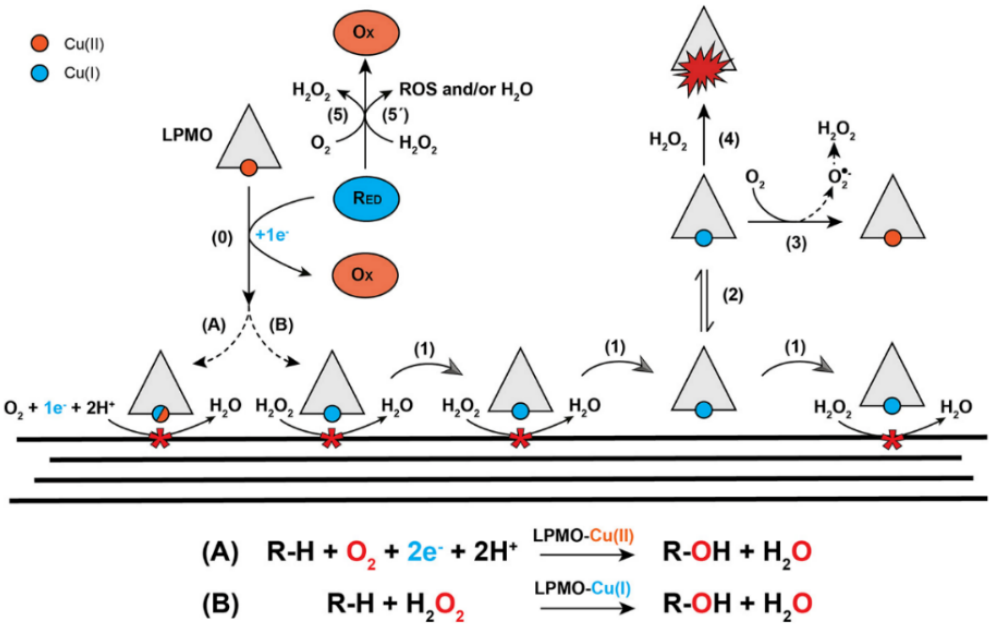


Figure 15. The monooxygenase (A) and peroxygenase (B) mechanisms, as well as other LPMO-related reactions (0 to 5'). *ROS, reactive oxygen species. The figure was derived from* (213) *under the CC BY 4.0 license. Note that the reactions are described in detail in the main text.*

Figure 15 depicts the many additional reactions that can take place (labelled as reactions 2 to 5') in addition to the main LPMO reaction that leads to scission of glycosidic bonds. These side-reactions substantially complicate practical work with LPMOs. Reaction 2 in Figure 15 depicts a situation where the reduced LPMO is not bound to the substrate. This may lead to a futile oxidase reaction (reaction 3), resulting in the production of H_2O_2, which can either drive the true peroxygenase reaction (reaction 1) or cause LPMO self-inactivation when present in excessive amounts (reaction 4). In reaction 5, the reductant itself reduces O_2, generating H_2O_2. When, instead, the reductant reduces H_2O_2 (reaction 5'), reactive oxygen species and/or water are produced. Both reaction 5 and 5' are affected by transition metals in solution.

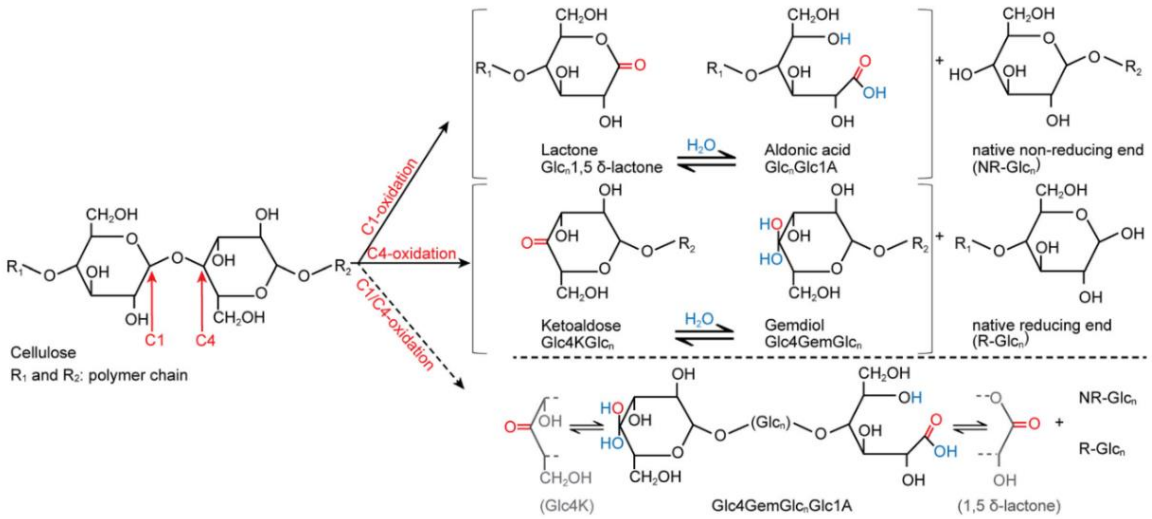


Figure 16. Possible C1-, C4-, and C1/C4-oxidized LPMO reaction products derived from cellulose. *Glc, glucose. The figure was retrieved from* (214) *under the CC BY 4.0 license.*

4.3 Structural features

X-ray crystallography remains one of the most accurate techniques to characterize protein structures. To date, 21 crystal structures of LPMO9s have been solved (according to the CAZy database as of November 2022), including structures of two C4-oxidizing LPMO9s in complex with a cellohexaose substrate (215). These two co-crystal structures are shown in Figure 17. A feature common to all LPMOs is the histidine brace in the catalytic site of the enzyme. It is composed of the first histidine residue in the polypeptide chain and another histidine that usually occurs between positions 70 and 80. The nitrogen atoms from the two histidine side chains, together with the N-terminal amino group, bind and position the Cu atom (the orange spheres in Figure 17) in what is usually a rather flat substrate-binding surface. In LPMOs produced by filamentous fungi, this *N*-terminal histidine carries a methylation that protects the LPMO from self-inactivation by reactive oxygen species (216). LPMOs generally have very high affinity (nM to pM) for Cu, and the dissociation constant K_d

for Cu(I) is lower than for Cu(II) (217). The active sites on LPMO9s contain characteristic residues also in the second copper coordination sphere; they include a tyrosine whose hydroxyl group occupies the proximal axial copper coordinating position, plus two conserved glutamine and histidine residues (218). Based on multiple sequence alignment and structural modelling, all *Tt*LPMO9s characterized in **Paper II** contained these residues, along with the histidine brace.

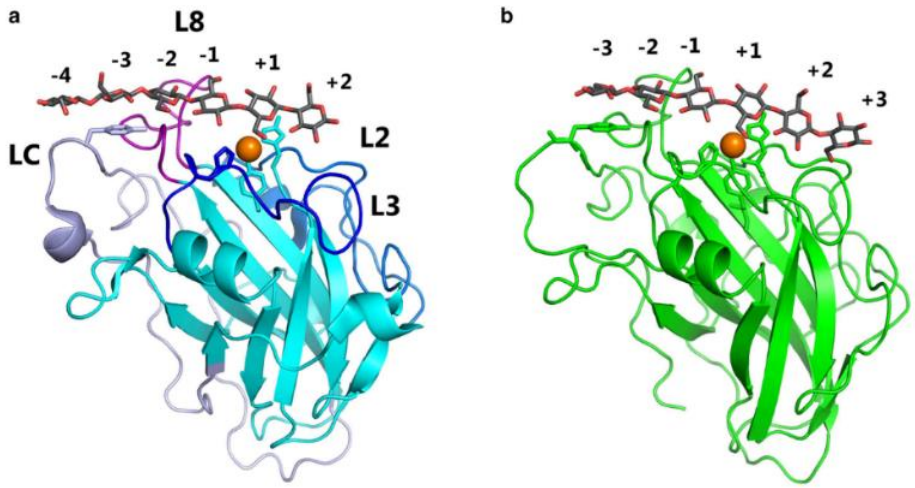


Figure 17. X-ray crystal structures of Lentinus similis LsLPMO9A (a) and Collariella virescens CvLPMO9A (b) forming a complex with cellohexaose (grey carbons). *The numbers indicate substrate-binding subsites. The Cu atom is shown as an orange sphere. For LsLPMO9A, substrate-binding loop C-terminal (LC), L2, L3, and L8 regions are marked. The figure was retrieved from* (190) *under the CC BY 4.0 license.*

Some LPMO9s carry a cellulose-binding CBM1 domain at the C-terminus, connected to the AA9 catalytic domain via a linker. In addition to CBM1s, other domains connected to AA9 catalytic domains have been reported based on genomic analysis (102) and can also be found by sequence analysis using Pfam (219). Courtade and colleagues showed that a CBM2 linked to the catalytic domain of a cellulose-active *Sc*LPMO10C directed the enzyme to a specific location, where it oxidized the cellulosic substrate (220). Such a mechanism augments the probability of releasing small soluble products. CBMs favor both LPMO9 binding and activity (135,137,212,221,222). Recently, disordered regions that are sometimes linked to the C-terminus of LPMO catalytic domains (in all families except AA13) have started to gather interest (223), but their function has not been demonstrated yet.

The core LPMO9 structure displays an immunoglobulin-G-like β-sandwich fold consisting of seven to eight β-strands that make up two β-sheets. Usually, this fold contains also two to three short α-helices (Figure 17); whereas two to three disulfide bridges normally stabilize the LPMO tertiary structure. The helices and sheets are separated by several disordered loops (for instance the loops marked L2, L3, LC, and L8 in Figure 17), which have been associated with substrate recognition. In LPMO9, the main determinants of substrate specificity are thought to be located in

the highly variable L2 loop region, which may include surface-exposed aromatic residues involved in substrate binding. Some LPMO9s include an L3 loop facing the L2 loop, but none of the LPMO9s described in **Paper II** displayed this L3 loop. Loop C-terminal (LC) and loop short (LS) regions are also typical of LPMO9s and may contain solvent-exposed aromatic residues that help shape the substrate-binding surface (218). Notably, one of the LPMO9s described in **Paper II**, *Tt*LPMO9U, which showed activity in both Amplex Red and 2,6-dimethoxyphenol (DMP) assays used for detecting redox-active copper contained a special loop at positions 214 to 224, which none of the other LPMOs in the study had (Figure 18). However, *Tt*LPMO9U was not active on any of the tested polysaccharides (**Paper II**). This extended loop might play a role in binding an unknown substrate, possibly suggested by its two glutamine residues, which are sometimes involved in substrate binding in e.g CBM1s (224).

The substrate-binding surface of LPMO9s is relatively flat (Figure 17 and 18), which contrasts with the substrate-binding clefts, or even tunnels, of canonical cellulases, but also with the more “rugged” substrate-binding surfaces of AA13, AA14, and AA17 family LPMOs (204). As indicated in Figure 17, an oligomeric substrate binds to LPMOs via hydrogen bonds, as well as via interactions with conserved aromatic residues. Nonetheless, the binding of polymeric (crystalline) substrates remains to be elucidated. Recently, a possible LPMO9 inhibitor called cinnamtannin B1 was identified by screening natural plant extracts (225); whereas fermented persimmon juice, used to preserve wood, has been shown to inhibit five LPMO9s and one LPMO10 (226). The discovery of LPMO inhibitors has important implications because these compounds may block LPMOs that act as virulence factors.

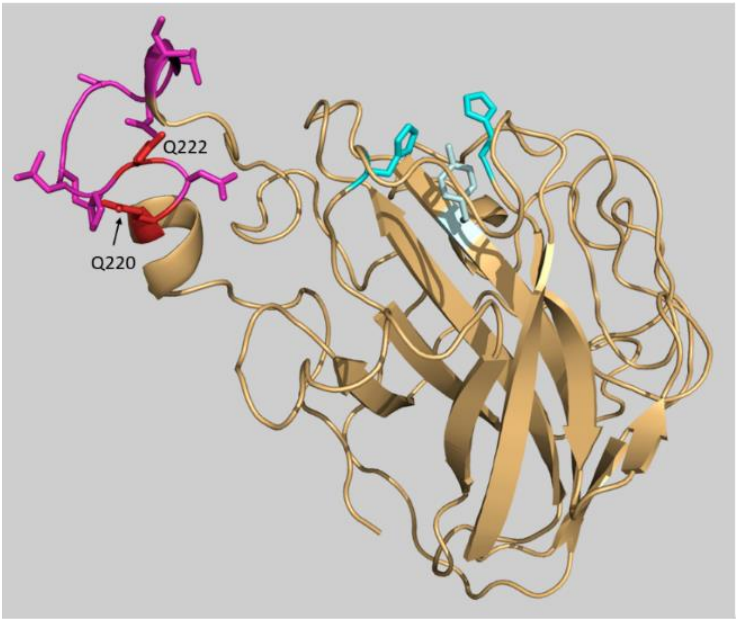


Figure 18. A Phyre2 model of TtLPMO9U illustrating the extra loop in the 214–224 region (in magenta). *The polar glutamine residues are colored in red and the histidine brace in cyan, whereas the Tyr in the proximal axial coordination position is colored in light blue. Modelling and figure courtesy to Dr Olav A. Hegnar.*

The most common recombinant LPMO9 production system is the yeast *Pichia pastoris*, which uses the inducible pAOX1 promoter or the pGAP constitutive promoter. Such a recombinant protein production system in yeast is relatively convenient for practical work, since usually high cloning success and protein yields are possible, resulting in active LPMOs with correctly processed signal peptides. Correct signal peptide cleavage is crucial for LPMOs, as the first residue (histidine) of the secreted and processed protein is involved in copper coordination. There are, however, a few disadvantages connected to use of such a heterologous yeast production system (227,228). First, *P. pastoris* is known for elevated *N*- and *O*-glycosylation of recombinant proteins, especially in linker regions (*O*-glycosylation), and the inability to methylate the first histidine may not protect LPMOs from self-inactivation. Second, glycosylation patterns likely differ between *P. pastoris* and native filamentous fungi. Thus, numerous characterized LPMO9s have likely been *N*- and/or *O*-glycosylated in a non-natural manner but the effect of such glycosylations has not been systematically studied. As reviewed by Gaber and colleagues, glycosylation may affect LPMO interactions with the substrate, protein solubility, and/or enzyme activity (228). In addition, in some cases such as *Tt*LPMO9B and *Tt*LPMO9U described in **Paper II,** LPMOs may have putative glycosylation regions near the active site, that may, when glycosylated, interfere with enzyme activity. However, as reported in **Paper II**, I was able to remove *N*-glycosylation modifications from *Tt*LPMO9U, but this was not sufficient to endow the enzyme with activity against the most common LPMO substrate, PASC.

An additional drawback of the *P. pastoris* system is background interference from endo-glucanases and/or endo-xylanases, for example from strains X33 and SMD1168H. Contaminating hydrolases are a common source of background signal in LPMO reactions involving reductant-independent cleavage of glycosidic bonds. For *Tt*LPMO9U and *Tt*LPMO9T described in **Paper II**, such background signal was unfortunately relatively high, even though sodium dodecyl sulfate polyacrylamide gel electrophoresis indicated that the enzyme preparation was homogeneous after one-step purification. Of note, similar background interference was detected in the work by Hüttner and colleagues (136), even though chemically very different two-step purification was used in that study compared to the purification method used in **Paper II**. The unknown nature of these contaminating cellulases and/or xylanases makes it nearly impossible to find inhibitors or specific reaction conditions to deactivate them. Accordingly, it is crucial to always include reductant-free controls when setting up LPMO reactions, as such high background activities might otherwise be ascribed to LPMOs. The fact that these background activities appear in LPMO samples prepared in very different ways (e.g., **Paper II** vs the work of Hüttner et al.), makes one wonder if this background activity could belong to LPMO9s themselves. In fact, endoglucanase activity was suggested for LPMO9s before 2010 and such suggestions have been reiterated in the literature in rare cases after that (229). However, most hydrolases possess conserved acidic amino acid residues in their catalytic sites, which are not found in LPMOs. This makes the hydrolytic nature of LPMOs highly unlikely, although it cannot be ruled out entirely as the hydrolytic activity might derive from the copper-containing active site.

4.4 Multiplicity and substrate specificity of AA9 LPMOs

AA9 LPMOs are the most functionally characterized LPMOs, likely due to their clear industrial relevance. At the time of writing this thesis (November 2022), the CAZy database listed 38 characterized LPMO9s (LPMO10s come second with 34 entries). This is probably an underestimation as, for example, the five novel LPMO9s described in **Paper II** have not yet been submitted to CAZy.

LPMO9s are common in fungi. After family AA11 LPMOs, AA9s are the most widespread in fungi. According to Varnai et al., 57.5% of sequenced basidiomycetes and 76.6% of sequenced ascomycetes encode one or more AA9s (230). On average, dikaryotic fungi harbor 12 AA9-encoding genes in their genomes (230). In fact, the basidiomycete *Rhizoctonia solani*, a plant pathogen, encodes as many as 55 AA9 genes (230). Knowing the connection between LPMOs and virulence factors, it is likely that some of them contribute to the fungus' pathogenic potential. Despite their extensive characterization, AA9 multiplicity, substrate specificity, and structure-function relationships remain to be determined.

LPMO9 activity has been demonstrated on numerous substrates: celluloses from various sources, hemicelluloses, and also respective oligomers (56,135–137,207,231). Notably, even though it has been continuously suggested and shown that LPMOs act on crystalline substrates such as cellulose (47,191,232), numerous AA9 LPMOs are even more active on amorphous/less crystalline cellulose (137,201,233,234). A similar tendency was observed for *Tt*LPMO9s discussed in **Paper II**. It should be kept in mind that the substrates used in **Paper II** and elsewhere are often extracted from their natural contexts and/or pretreated in multiple ways, thus masking the true activity on native substrates. All in all, it has become evident that some AA9s show high activity on other polysaccharides besides crystalline cellulose.

LPMO substrate specificity is commonly analyzed by the combination of two methods (also in the studies described in **Papers II** and **III**): high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). Hydrophilic interaction liquid chromatography with electrospray ionization MS has also been employed, especially when unraveling C1/C4/C6 regioselectivity (209,235). In all these methods, the analytes are soluble products, and most conclusions regarding LPMO activity are drawn based on their release.

HPAEC-PAD relies on the ionizability of carbohydrates assuming an ionized form at high pH and a sensitive electrochemical detector. The method successfully distinguishes native and oxidized monosaccharides and oligosaccharides, making it highly suitable for analyzing oxidized LPMO products directly. Use of HPAEC-PAD and MALDI-TOF in LPMO research has been reviewed extensively by Westereng et al. (205,229). The analytes in MALDI-TOF MS are crystallized together with a matrix compound, and are then desorbed and ionized under a laser beam. The ions

are detected based on their “time of flight”, which relates to their mass-to-charge ratio (m/z), when accelerated towards the detector (236). Although there are numerous other ways to assess LPMO activity, HPAEC-PAD (both quantitative and qualitative analysis) and MALDI-TOF MS (qualitative analysis) have remained the most popular in the field. This could be ascribed to the availability of these instruments in chemistry and biotechnology laboratories, as well as their relative ease of use.

Following the expression of recombinant proteins, LPMO activity may be assessed using either the Breslmayr/2,6-DMP assay (237,238) or the Kittl/Amplex Red assay (239). A combination of HPAEC-PAD, MALDI-TOF, 2,6-DMP assay, and Amplex Red assay was used in the study described in **Paper II**.

From both an industrial and research perspective, it is important to understand the reason for the multiplicity of (AA9) LPMO genes and the enzymes they encode. This would enable us to fully unravel the roles of AA9s in nature, but also to find enzymes with new functionalities and improved stability for industrial use. However, systematic functional characterization of LPMO9s has remained scarce as there is no standard protocol for it. The filamentous fungi, whose LPMO9 substrate specificity has been most studied include *Podospora anserina* (137), *Neurospora crassa* (135,231)*, M. thermophila* (240,241), and *Malbranchea cinnamomea* (136)*.*

In 2015, Bennati-Granier and colleagues studied the substrate specificity of five *P. anserina* AA9 LPMOs and the oxidative regioselectivity of three out of 33 LPMO-encoding genes. Three CBM1-containing *Pa*LPMO9s were highly active on PASC, but only one of them showed some activity on cellooligosaccharides, carboxymethylcellulose, barley β-glucan, glucomannan, lichenan, and xyloglucan (137). Four LPMO9s (of eight in total) from *M. cinnamomea* were systematically tested on numerous substrates by Hüttner and colleagues (136). One of the *Mc*LPMO9s showed medium activity on konjac glucomannan and, together with *Mc*LPMO9F, was also active on cellohexaose. Interestingly, three of the four *Mc*LPMO9s were active on pure TXG, whereas the fourth enzyme, *Mc*LPMO9H, required the addition of PASC. The other three *Mc*LPMO9s showed TXG activity also on a cellulose-TXG co-polymeric structure. Notably, *Mc*LPMO9H showed xylanolytic activity on PASC, which contains traces of xylan, and the preference of this enzyme for xylan was further explored by experiments with mixtures of PASC and beech glucuronoxylan. The three other *Mc*LPMO9s showed minor xylanolytic activity. The genome of the filamentous fungus *N. crassa*, a common bread mold, encodes 14 LPMO9s. For most of these activity on cellulose has been demonstrated, whereas a broad analysis of the substrate specificities of *Nc*LPMO9s has been rather fragmented in different studies (135,207,231). Petrovic and colleagues characterized systematically three *Nc*LPMO9s, and all showed activity on both TXG and konjac glucomannan. Interestingly, in some cases, the addition of PASC as a cellulose matrix to TXG and konjac glucomannan promoted or disclosed the activity of *Nc*LPMO9 on these hemicelluloses (135). Hegnar and colleagues

proved that *Nc*LPMO9F possessed high xylanolytic activity and *Nc*LPMO9L low xylanolytic activity on xylan-PASC mixtures (242). The genome of the fungus *M. thermophila* encodes 22 putative LPMO9s. The substrate specificity of *M. thermophila* LPMO9s has been tested heavily but in a rather fragmented way as well. The substrate specificity of *Mt*LPMO9s has been best summarized in the PhD thesis of Dr Peicheng Sun (243), who concluded that nine partially characterized *Mt*LPMOs exhibited cellulolytic activity. Additionally, three *Mt*LPMO9s have hemicellulolytic activity on cellulose-adsorbed xylan, xyloglucan, mixed-linked glucans and, in one case, cellooligosaccharides.

The abovementioned studies have generated substantial evidence on substrate specificity and multiplicity of LPMO9s, which point to functional variability among LPMO9s in filamentous fungi. However, a systematic and comparative functional screening of AA9s from the same species was still lacking. This led me to the work described in **Paper II**, in which I set out to determine whether the multiplicity of AA9 genes in filamentous fungi relates to functional variability, and whether novel AA9 activities could be discovered. Indeed, several functional differences were found between the six *Tt*LPMO9s used in this study, including previously unknown functionalities.

The experiments described in **Paper I** showed that 14 out of the 18 AA9 LPMO-encoding genes of *T. terrestris* LPH172 were transcribed on Avicel, beechwood xylan, and rice straw. Nine of these genes were selected for cloning and subsequent functional characterization (Table 1). Of note, as described in **Paper II**, only six of the nine selected recombinant LPMOs were successfully expressed in *P. pastoris.* It remains unclear why the success rate of LPMO cloning and protein secretion efforts is so low, and this is an interesting issue for future research. For instance, if mistakes have been introduced into protein coding DNA sequences during sequencing of fungal genomes, a gene with a mistake will be cloned, which can lead to altered protein translation, folding and/or secretion. Additionally, the redox LPMO chemistry may regulate their production in the host organisms.

Table 1. Overview of TtLPMO9 genes upregulated during growth on Avicel, rice straw, and beech xylan (Paper I) and subsequently functionally characterized (Paper II).

Name	Gene ID	UniProt ID	TPM A	TPM RS	TPM BX	Log2 FC A	Log2 FC RS	Log2 FC BX	Prod	AR	2,6-DMP	S
***Tt*9A**	TT08370	G2R6N0	8271	462	21	11	10	3	Y	Y	Y	C,XG,X
***Tt*9B**	TT04350	G2RB73	4537	9	13	10	3	1	Y	Y	Y	C
***Tt*9G**	TT01736	G2QZK6	2677	30	20	13	9	6	Y	Y	Y	C,XG,X
Tt*9E	TT07456	G2RGE5	8862	963	9	11	10	1	Y*	Y	Y	C,XG,X
***Tt*9J**	TT03770	G2QQL2	41	117	4	2	6	-	N	N/A	N/A	N/A
***Tt*9L**	TT06268	G2R898	7	47	4	6	11	5	N	N/A	N/A	N/A
***Tt*9M**	TT09068	G2QV07	-	9	-	6	13	3	N	N/A	N/A	N/A
***Tt*9T**	TT07455	G2RGE6	8	7	-	4	6	-	Y	Y	Y	C, XG
***Tt*9U**	TT04352	G2RB72	181	234	3	5	8	-	Y	Y	Y	N/A

The LPMOs were named according to (131)*. The Gene ID is according to Paper I. TPM, transcripts per million; Log2FC, log2 fold change; A, Avicel; RS, rice straw; BX, beechwood xylan; Prod, recombinant production (Y for yes and N for no); AR, activity in the Amplex Red assay; 2,6-DMP, activity in the 2,6-DMP assay (in both: Y for yes and N/A for not available); S, substrate specificity; C, cellulose; XG, xyloglucan; X, xylan associated with PASC. Downregulated genes or genes for which no differential expression was detected or where upregulation was not significant are indicated with "-". TtLPMO9E is marked with an asterisk as it was included in Paper II, while the recombinant strain was provided by Dr Anikó Várnai. For further details, please see Paper I and Paper II.*

Phylogenetically, the selected *Tt*LPMOs in **Paper II** were close to each other, compared to LPMO9 selection in the *Pa*LPMO9 screening (137) or the *Mc*LPMO9 screening (136). Sequence similarity between the six chosen *Tt*LPMO9s was still rather low (23%–53%), but all six *Tt*LPMO9s showed peroxidase-like activity in the 2,6-DMP assay and oxidase-like activity in the Amplex Red assay. Only five *Tt*LPMO9s were active on at least one of the tested carbohydrates, and all showed C1-oxidizing activity on cellulose. Of note, *Tt*LPMO9U showed relatively high activity in the 2,6-DMP assay, even though it was inactive on all tested polysaccharide substrates. Given that 2,6-DMP is a phenolic, lignin monomer-like compound, this result might be an indication of high affinity to lignin-like monomers, or it might also just be a coincidence related to the redox potential of the enzyme or the binding specificity and shape of the active site. However, due to the extra loop of this enzyme, indicated in Figure 18, it serves as an interesting candidate for possible future work aimed at finding new LPMO9 specificities.

All tested substrates and detected activities described in **Paper II** are summarized in Table 2. The most active LPMO on both cellulosic and hemicellulosic substrates was the well-known *Tt*LPMO9E. It was studied already in 2010 by Harris et al. and it was the second LPMO9 (GH61) for which a structure was published (156), as described in subchapter 4.1. *Tt*LPMO9E exhibited high activity on TXG, acetylated birch glucuronoxylan, and beech glucuronoxylan (**Paper II**), all in combination with PASC. Unsurprisingly, xylan activity could only be detected in reactions that also contained cellulose, confirming earlier reports of such cellulose dependency (136,240,242). It is tempting to assume that xylan interacts with cellulose,

generating a structure that resembles the co-polymer of these carbohydrates in native plant cell walls. As mentioned in Chapter 2, xylan adopts a more rigid and flattened two-fold conformation when combined with cellulose. As LPMO9s prefer flat substrates due to the shape of their active site, it is reasonable that such a conformation is needed for productive binding of LPMO9s.

Table 2. Summary of the activities shown by six TtLPMO9s described in Paper II.

Substrate	*Tt*LPMO9A	*Tt*LPMO9B	*Tt*LPMO9E	*Tt*LPMO9G	*Tt*LPMO9T	*Tt*LPMO9U
PASC	+++	+	+++	+++	+	–
Avicel	++	+	+++	+++	+	–
Pulp fibers	++	+	+++	+++	+	–
Glc_5	–	–	–	–	–	–
PASC+KGM	–	–	–	–	–	–
PASC+acGGM	–	–	–	–	–	–
PASC+WAX	–	–	–	–	–	–
PASC+TXG	+++	–	+++	+	(+)	–
TXG	(+)	–	(+)	(+)	(+)	–
PASC+BeWX	+	–	+++	+	–	–
BeWX	–	–	–	–	–	–
PASC+AGX	–	–	–	++	–	–
AGX	–	–	–	(+)	–	–
PASX+acGX	+++	–	+++	–	–	–
acGX	–	–	–	–	–	–

[a]Activity is indicated: (+), minor (trace activity); +, low; ++, medium; +++, high; –, absent. These are rough, not truly quantitative assessments that give an overall impression of the variation in substrate specificity. For each substrate, +++ stands for the maximum observed product level for that substrate.

Note that, in all cases, control reactions without reductant were carried out and activity is only scored in this table if the LPMO reaction with reductant showed oxidized product formation clearly above the background level. Glc, glucose; KGM, Konjac glucomannan; acGGM, acetylated galactoglucomannan; AGX, arabinoglucuronoxylan; acGX, acetylated birch glucuronoxylan; BeWX, beech glucuronoxylan; The table was retrieved from the paper under the CC BY 4.0 license.

HPAEC-PAD analysis revealed fewer products when PASC+TXG served as substrate for *Tt*LPMO9E compared to *Tt*LPMO9A, pointing to differences in substrate preferences. MALDI-TOF MS showed that *Tt*LPMO9E products included multiples of three pentose units, indicating that *Tt*LPMO9E was less tolerant than *Tt*LPMO9A to xylosyl substitutions adjacent to the scissile glycosidic bonds in TXG. In contrast, *Tt*LPMO9A generated a less defined product profile and a larger number of different products. Both LPMOs are unimodular, meaning that the tolerance of substitutions is tightly related to the catalytic LPMO9 domain, and most likely to differences among binding residues in the active site. *Tt*LPMO9A, which generated only C1-oxidized products when acting on cellulose, released C1/C4 double-oxidized products when using TXG as substrate, confirming the substrate-dependent oxidative regioselectivity of some LPMOs (212). When combined with PASC, *Tt*LPMO9E was highly active on acetylated birch glucuronoxylan and beech glucuronoxylan. *Tt*LPMO9G was active on all cellulosic substrates but showed

minor activity on PASC+TXG. However, in the work described in **Paper II** this LPMO was the only one capable of degrading (cellulose-associated) arabinoglucuronoxylan from spruce. As described in **Paper II**, *Tt*LPMO9G activity on arabinoglucuronoxylan could only be detected using HPAEC-PAD but not MALDI-TOF MS. *Tt*LPMO9A showed high activity on PASC, but was less active on more crystalline substrates, such as Avicel and pulp fibers. Besides tolerating xyloglucan substitutions, *Tt*LPMO9A was active on acetylated birch glucuronoxylan mixed with PASC. To the best of my knowledge, LPMO9 activity on (cellulose-associated) acetylated birch glucuronoxylan was demonstrated for the first time in **Paper II**. *Tt*LPMO9T seemed to be more active on crystalline cellulose substrates, such as Avicel and pulp fibers compared to PASC, although the absolute product levels were still relatively low. *Tt*LPMO9T showed only minor activity on TXG and TXG+PASC. *Tt*LPMO9B exhibited very low activity on all cellulosic substrates. None of the tested LPMOs were active on cellopentaose, various (galacto)glucomannans or arabinoxylan from wheat. However, in subsequent studies, additional xylanolytic activities of *Tt*LPMO9s were discovered (**Paper III**), as discussed in the following sub-chapter. All in all, the results summarized in Table 2 and described in **Paper II** show that the tested *Tt*LPMO9 enzymes display different activity levels and substrate preferences, corroborating the notion that the multiplicity of LPMO9 genes in filamentous fungi relates to functional variability. The previously unknown LPMO9 activities on native xylans from spruce and birch will likely favor the use of these polysaccharides in wood-based biorefineries.

It is worth noting that all LPMOs mentioned in **Paper II** were tested in standard conditions, at pH 6.5 and 40°C (244), with 1 mM gallic acid as reductant. It is possible that other substrate specificities and/or activities would be obtained if another pH, temperature, or reductant was used. In fact, proper pH and temperature optima measurements are rather rare in the LPMO literature, and it would be interesting to study this further. Additionally, it would be very interesting to study the activity of *Tt*LPMO9s on insoluble products. It is possible that LPMO9s, which released only a limited amount of soluble products, nevertheless possessed significant activity that remained undetected since only soluble products were analyzed. This notion relates to the work described in **Paper V** and will be covered further in the next chapter.

4.5 Xylanolytic activity of AA9 LPMOs

The activity of LPMO9s on hemicellulosic substrates has been known since the 2014 landmark study by Agger et al., in which reductant-dependent activity of *Nc*LPMO9C on xyloglucan, mixed-linked β-glucan, and glucomannan was demonstrated (231). A combination of glycan microarray technology, MS, and chromatographic methods confirmed that the observed activity was due to LPMO

and not from background enzyme contamination. Elucidation of hemicellulolytic capability by LPMO9s is highly relevant as the retention of hemicelluloses in feedstocks can block cellulase access to cellulose (e.g. in (224–226)). LPMO9s might help relieve this inhibition, as discussed in detail in Chapter 5.

The work of Agger and colleagues was followed by the work of Frommhagen et al., in which the authors for the first time showed that *Mt*LPMO9A cleaved xylan in a reductant-dependent reaction, using both HPAEC-PAD and MALDI-TOF MS (240). Notably, this study also showed that for the LPMO9 to be active on xylan, the latter had to be mixed with cellulose. The authors tested birchwood glucuronoxylan, oat spelt arabinoxylan, and wheat arabinoxylan in combination with regenerated cellulose, but xylanolytic activity of *Mt*LPMO9A was detected only for the two first xylan substrates. In 2017, Simmons and colleagues studied *Ls*LPMO9A and *Cv*LPMO9A, and concluded that the former cleaved isolated xylan and xylohexaose in a reductant-dependent manner (56). However, the authors only used carbohydrate gel electrophoresis to analyze products, thus failing to detect oxidized xylooligomers. The authors were unable to detect oxidized xylooligomers using mass spectrometry. These results imply that some LPMO9s could degrade pure xylans in solution, but additional data seems necessary to substantiate this.

In 2019, Hüttner and co-authors noted that *Mc*LPMO9H was producing reductant-dependent oxidized xylooligomers from PASC (136). The xylanolytic activity of this enzyme was probed further by MS using beechwood glucuronoxylan. These additional analyses confirmed that *Mc*LPMO9H possessed xylanolytic activity and preferred xylan over cellulose when acting on a cellulose-xylan mixture. Of note, in the same year, a xylanolytic LPMO10 was reported (163). However, the detected activity was not reductant-dependent and the authors did not present convincing MALDI-TOF MS data to support the HPAEC-PAD results. Hegnar and colleagues (242) showed that *Nc*LPMO9F, which phylogenetically clusters together with *Mc*LPMO9H, preferred cleaving xylan (the authors reported a 3:1 ratio for xylan:cellulose degradation products) when presented with a xylan-cellulose mixture. Importantly, oxidized xylanolytic products were quantified for the first time in this study. This was achieved using an endo-xylanase that converted oxidized xylooligomers to xylobionic and xylotrionic acid, which were then quantified by HPAEC-PAD. Based on structural and phylogenetic analyses, the aromatic tyrosine 2 and 71 residues were associated with the xylanolytic activity of LPMO9s.

As the three xylanolytic *Tt*LPMO9s described in **Paper II** were found to be active on glucuronoxylan, acetylated glucuronoxylan, and arabinoglucuronoxylan, I was curious why there was no apparent activity on wheat arabinoxylan. The latter had a 62:38 xylan:arabinose ratio, which made it the most substituted xylan used in the work of **Paper II** (see Table 3). As detailed in **Paper III**, this realization made me wonder whether the abundant xylan substitutions hindered LPMO9 action.

Table 3. Substitution of the xylan substrates used in Paper II and Paper III.

	Xyl %	Ara %	MeGlcA %	Ac %	Fraction of substituted xyloses	Reference
WAX38	62	38	0	0	0.61	Megazyme
WAX30	70	30	0	0	0.43	Megazyme
WAX22	78	22	0	0	0.28	Megazyme
SpAGX	70.6	10.7	12.4	0	0.33	(63)
acGX	74.8	0	6.7	30	0.49	(63)

WAX, wheat arabinoxylan; SpAGX, spruce arabinoglucuronoxylan, acGX, acetylated birch glucuronoxylan; MeGlcA, methylglucuronic acid; Ac %, degree of acetylation; Xyl, xylose; Ara, arabinose. The number after WAX indicates the percentage of arabinose. Note that for the WAX substrates, it is not possible to say if the percentages indicate mass or mol. For spruce and birch xylans, the percentages for monosaccharides indicate mol %. Both acGX and SpAGX contained other residual monosaccharides not shown in this table but listed in the original study. Also note the lack of information on the substitution level of commercial glucuronoxylan from beechwood (Apollo Scientific).

Indeed, activity assays with commercially available wheat arabinoxylan substrates characterized by different substitution levels revealed reductant-dependent *Tt*LPMO9E activity on arabinoxylan when the arabinose content was lowered to 22%, as indicated by HPAEC-PAD (Figure 19, **Paper III**) and MS (**Paper III**)**.** All xylanolytic activities described in **Paper III** were detected only when using xylan-cellulose mixtures.

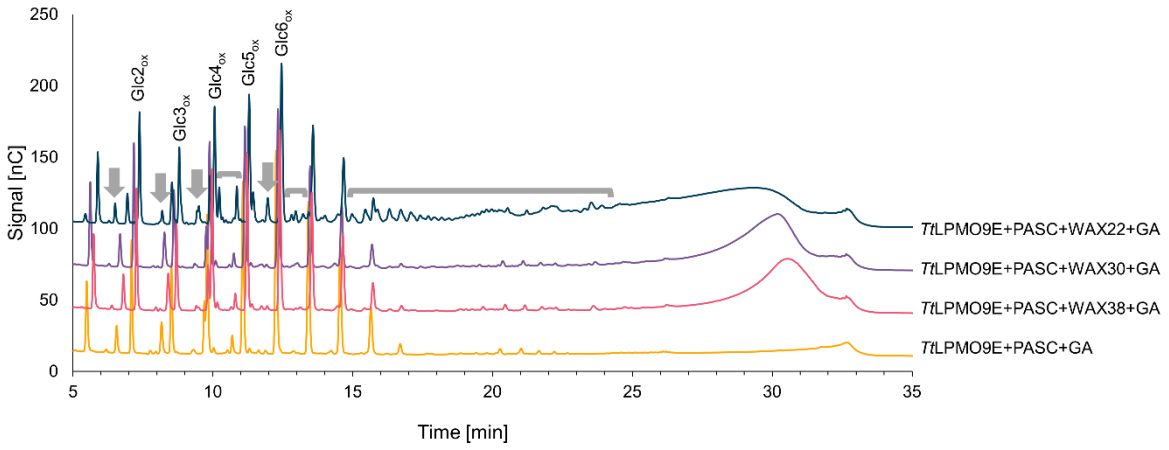


Figure 19. TtLPMO9E activity on PASC+WAX22 analyzed by HPAEC-PAD. *Only the uppermost green chromatogram shows xylan-derived products (labelled by grey arrows and brackets), demonstrating reductant-dependent TtLPMO9E activity on WAX22+PASC. The purple and pink chromatograms show lack of TtLPMO9E activity on WAX30+PASC and WAX38+PASC, respectively. The orange chromatogram is used as a reference for TtLPMO9E-generated cellulose-derived products. Gallic acid (GA) was used as reductant. The figure was retrieved from Paper III under the CC BY 4.0 license.*

By employing xylan-debranching enzymes such as a GH62 family arabinofuranosidase, I was able to confirm that debranching of both WAX38 and WAX22 promoted the xylanolytic activity of *Tt*LPMO9E. Furthermore, a combination of the GH62 and a GH115 (methyl)glucuronidase successfully debranched spruce arabinoglucuronoxylan, demonstrating for the first time *Tt*LPMO9E activity on this substrate (Figure 20). This finding was rather remarkable as *Tt*LPMO9E has been studied extensively since 2010.

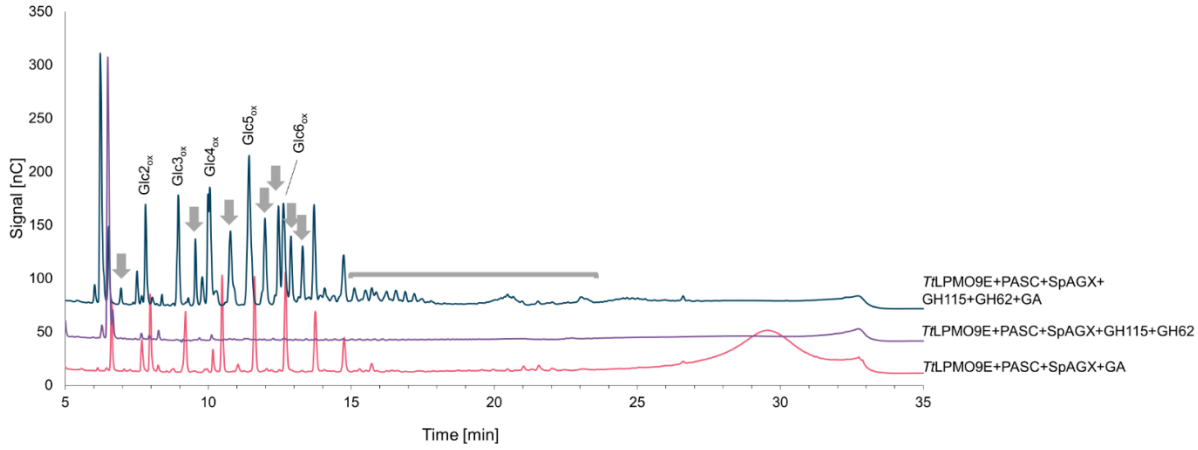


Figure 20. Reductant-dependent TtLPMO9E activity on PASC+spruce arabinoglucuronoxylan (SpAGX) in which the SpAGX was debranched with GH62 and GH115*. Only the top green chromatogram shows xylan-derived products (labelled by grey arrows and brackets), demonstrating reductant-dependent TtLPMO9E activity on debranched SpAGX+PASC. The purple chromatogram shows the reductant-free control and the pink chromatogram indicates no xylanolytic TtLPMO9E activity on PASC+SpAGX in the absence of GH62 and GH115. The figure was retrieved from Paper III under the CC BY 4.0 license.*

The study described in **Paper III** reports that the xylanolytic activity of *Tt*LPMO9E was hindered by the abundant arabinose substitutions on wheat xylan and that for *Tt*LPMO9E activity to become noticeable the arabinose substitution level must fall to somewhere between 22 and 30%. *Tt*LPMO9G activity on arabinoglucuronoxylan from spruce was boosted by debranching enzymes. Interestingly, in tests where Avicel was used instead of PASC as the cellulose matrix, *Tt*LPMO9E activity on both cellulose and xylan was hampered. The reason for this remains unknown, although it might relate to the small surface area of Avicel (57). It also remains to be determined if the beneficial effect of debranching enzymes on LPMO9 activity comes from improved xylan adsorption onto cellulose. As explained in subchapter 2.2, xylan debranching can improve xylan interaction with cellulose. It is plausible that debranching simply alters the substrate-binding surface and thus removes steric hindrance from LPMO9s. Alternatively, the debranching effect may depend on the chemical nature of the substitutions (e.g., bulky methyl glucuronic acid vs. smaller acetyl/arabinose), specific substitution pattern (even vs. non-even), and

varying substrate-binding surface of LPMOs. In fact, some LPMOs may need certain substitution patterns for substrate recognition.

Paper III shows the importance of choosing the right components when conducting functional screenings of LPMOs. The combined work described in **Papers II** and **III** indicates that, by exploiting commercially available substrates, we might be overlooking important LPMO activities. Native lignocellulose exists as a complex composite structure, whose features may not be captured in commercially available substrates. The work described in **Paper III** highlights the importance of trying to mimic natural conditions as closely as possible when screening LPMO activity *in vitro*. Interestingly, the results described in **Paper I** show that a GH62 arabinofuranosidase-encoding gene was highly expressed and upregulated together with multiple LPMO9 genes (Figure 14). Accordingly, the two enzyme classes might work synergistically also in nature, although further research should confirm the biological role of this partnership. In the next chapter and **Paper IV**, I explain how the xylan-degrading ability of LPMO9s may play an important part in the enzymatic saccharification of xylan-containing lignocellulosic biomass.

5. Applications of AA9 LPMOs

In this Chapter, I describe how LPMO9s can be utilized in biomass saccharification and functionalization and/or to produce nanocellulosic materials. I begin this Chapter by describing the suggested roles of LPMO9s in lignocellulolytic cocktails. The reaction conditions for optimal LPMO9 activity during saccharification are relatively intricate, and in subchapter 5.2 I therefore explain the main factors contributing to this complexity and how they relate to my work in **Paper IV**. I also present the results of **Paper IV** which show that LPMO9s aid in degrading very recalcitrant feedstocks like mildly pretreated spruce. Furthermore, to put my work in **Paper V** into context, I introduce CNCs and CNFs and summarize the relatively scarce literature describing previous attempts to use LPMO9s to functionalize sulfated CNCs. I thereafter present the results of **Paper V** showing how me and co-authors was able to successfully use LPMO9s for this purpose.

5.1 AA9 LPMOs in lignocellulolytic cocktails

Novozymes contributed to the discovery of LPMO9s while looking for enzymes with novel activity to improve their enzymatic cocktails, as described in subchapter 4.1 (134,154,156,196). Today, the most studied application of LPMO9s is in (ligno)cellulolytic enzyme cocktails. Two such cocktails from Novozymes, Cellic CTec2 and CTec3, contain LPMO9s (248); whereas their Celluclast cellulase cocktail contains no or very low LPMO activity (**Paper IV**) (140,192). Cellic CTec2 and CTec3 likely contain additional improvements compared to Celluclast and not just the LPMO9 addition. The application sheet for Cellic CTec2 mentions “aggressive cellulases”, abundant β-glucosidases, and a hemicellulase (249). Instead, the application sheet for CTec3 describes the inclusion of advanced LPMO9s, improved β-glucosidases, and hemicellulases (250).

To directly test the effect of LPMO addition to lignocellulolytic enzyme cocktails, spiking of the benchmark cocktail Celluclast with LPMOs is commonly done (**Paper IV**) (115,161,162,188). As shown in these cited studies, replacing 5%–20% of the total protein loading of a benchmark cellulase cocktail with LPMO9s can increase saccharification yields with both pure cellulose such as Avicel and variously pretreated lignocellulosic materials, such as sulfite-pulped spruce and steam-exploded birch. The enhancing effect depends closely on the substrate and process conditions, as discussed in the next subchapter. Cellic CTec2 is generally more efficient at biomass saccharification compared to Celluclast + Novozym 188 (140,192,194), which can be ascribed at least in part to the activity of LPMO9s in Cellic CTec2. Note that these studies have focused only on the release of cellulose-derived products.

5.1.1 The role of AA9 LPMOs in lignocellulolytic cocktails

It has become common to state that cellulose active LPMOs attack primarily crystalline areas and create attack sites for cellobiohydrolases in the cocktails, although it remains to be seen if this explains the enhancing effect of LPMO9s. In the work shown in **Paper IV**, I and co-authors showed that *Ta*LPMO9A reduces the cellulose crystallinity of mildly pre-treated spruce. Eibinger et al. have shown that crystalline areas attacked by cellobiohydrolases are first recognized by an *Nc*LPMO9. Such enzymatic synergy was shown to be most prominent on crystalline cellulose (232). Hu et al. reported a good correlation between the crystallinity of the substrate and the impact of LPMO9s on the saccharification efficiency of a cellulolytic enzyme cocktail (191). Simulations by Vermaas et al. indicated that LPMOs might help reduce crystallization, creating new termini for other cellulases. Importantly, these authors also pointed out that LPMO action might relieve product inhibition of cellobiohydrolases (252). Additional real-time imaging showed that *Tr*LPMO9A helped to separate fibrils in bacterial cellulose (253). Villares and colleagues reported that *Pa*LPMO9H acted on accessible amorphous cellulose, and the related chain cleavage and chemical modifications weakened the cellulose structure (233). In similar work, *Pa*LPMO9E was shown to oxidize fiber surfaces, without reducing their crystallinity, even though LPMO-treatment improved mechanical cellulose fibrillation (254). Addressing the aspect of LPMO-cellobiohydrolase synergy, Keller et al. have shown that C1-oxidizing LPMO9s block cellobiohydrolases and promote, instead, the activity of endoglucanases (255,256). Tokin and colleagues disclosed possible negative effects of LPMOs on certain cellulases by showing that they were LPMO- and substrate-dependent (234). Generally, it is important to note that cellulose-acting mechanisms of LPMO9s may be enzyme-dependent and cannot be generalized. Interestingly, ascorbic acid alone can boost cellulase activity, and this effect might contribute to the apparent enhancing effect of LPMOs (257). In the study described in **Paper IV**, we used wide-angle X-ray scattering to show that *Ta*LPMO9A reduced the crystallinity of mildly pre-treated spruce substrates with least or no hemicellulose. In summary, while the role of LPMO9s in lignocellulolytic cocktails remains to be elucidated, **Paper IV** confirms the hypothesis that at least some LPMO9s reduce the crystallinity of cellulose and likely make the substrate less recalcitrant for other cellulases by doing so.

5.1.2 Possible role of AA9 LPMOs in removing recalcitrant hemicelluloses

Current biorefining trends favor mild biomass pretreatments, as they lower energy consumption (248) and limit the loss of polysaccharides from chemical degradation (67). Hemicelluloses that are retained in the feedstock together with cellulose further complicate the saccharification processes as they can be highly recalcitrant and cover cellulose (248). Indeed, substrates containing hemicelluloses are more recalcitrant to enzymatic hydrolysis (247). Accordingly, addition of hemicellulolytic enzymes increases saccharification yields, confirming how hemicelluloses limit substrate accessibility for cellulases (245,246,258). The discovery of LPMO9

activity on xylans (**Papers II**, **III** and additional experiments with *Ta*LPMO9A related to **Paper IV**) and glucomannans may open new routes towards enzymatic removal of hemicellulose. In subchapter 5.3 I will describe how the xylan-degrading ability of AA9s might help increase xylose release (in addition to glucose release) during saccharification (**Paper IV** and related additional experiments).

5.2 LPMO reaction conditions and other factors to be considered

The contribution of LPMO9s to the efficiency of lignocellulolytic enzyme cocktails is known, but the inclusion of LPMOs requires reconsidering process conditions, particularly given the peroxygenase activity of these enzymes (12,27). Below I will explain some of the most important aspects that need to be considered when studying and/or applying LPMOs in lignocellulolytic cocktails (in no specific order).

5.2.1 Hydrogen peroxide

Hydrogen peroxide feed or spiking can substantially improve biomass saccharification yields when using LPMO-containing cocktails (187,216,251). For instance, the benefit of controlled H_2O_2 feeding for final sugar release from sulfite-pulped spruce substrate was shown with Cellic CTec3 at demonstration scale (2000 L) (188). This evidence suggests possible scale-up to industrial settings. Lignin can also provide H_2O_2 (195), along with LPMO itself (213,259) or even other oxidoreductases, such as cellobiose dehydrogenase (93) and glucose oxidase (94,181). However, H_2O_2 feeding should be accurately controlled and kept within low levels. Moreover, it benefits mostly the degradation of lignin-poor substrates, likely because of the side reactions that occur between lignin and H_2O_2, which release detrimental reactive oxygen species that inactivate LPMOs and other enzymes in the cocktails. This phenomenon may be mitigated by adding catalase to scavenge excess H_2O_2, as demonstrated even before the peroxygenase mechanism was known (260). Interestingly, transcriptome data in **Paper I** revealed the simultaneous upregulation of a catalase-peroxidase gene and LPMO9-encoding genes in *T. terrestris* LPH172, but only on beech xylan (Figure 14). Notably, measuring oxidation-reduction potential offers a potential tool to control H_2O_2 feeding (261,262). Owing to the many unknowns related to H_2O_2 feeding on lignin-containing substrates, we did not study this strategy in **Paper IV**, although it would be of great interest to do so in the future. Importantly, we showed that improved aeration through greater reaction headspace did not increase yields, indicating that the reactions described in **Paper IV** were not inhibited by lack of co-substrate generation.

5.2.2 Electron donor

An additional important aspect of LPMO reaction conditions concerns the electron donor (explained in subchapter 4.2). From an industrial point of view,

supplementation with an external reductant, such as ascorbic or gallic acid, adds unwanted costs. Various lignins or lignin-derived compounds, which are present in most industrially used substrates, can serve as electron donors (191,193–195). For example, lignosulfonates are good reductants for the saccharification of sulfite-pulped spruce (192). In many cases, oxidation of reductants by molecular oxygen can lead to the generation of the co-substrate H_2O_2 (195,202,213), thereby facilitating LPMO reactions that may be limited by insufficient H_2O_2. In the study described in **Paper IV**, addition of ascorbic acid failed to enhance saccharification yields, and actually decreased them in some cases. Specifically, 10 mM ascorbic acid was chosen based on reports and experiments showing that high ascorbic acid concentrations yielded the best results (**Paper IV**) (187). These experiments were performed with pure (i.e., reductant-free) cellulosic substrates, meaning that ascorbic acid likely drove *in situ* production of H_2O_2. However, 10 mM ascorbic acid was probably too much when applied to the redox-active lignin-containing substrates described in **Paper IV**.

5.2.3 Oxidized end-products

The final ratio of native monosaccharides to oxidized sugars after saccharification is also of economic relevance, because yeasts used for subsequent fermentation of the released sugars cannot utilize the oxidized form (263). Cannella et al. reported that oxidized products obtained after saccharification of pretreated wheat straw by Cellic CTec2 comprised 4% of final sugars (194). On the other hand Rodríguez-Zúñiga et al. reported that this was less than 1% when Cellic CTec2 was used to saccharify differently pretreated sugarcane bagasse (264). For the work described in **Paper IV**, Novozymes kindly provided us with *Ta*LPMO9A, a well-studied LPMO9 with prominent C4-oxidizing activity (140). Interestingly, even though initial studies detected C1-oxidizing LPMO activity in Cellic CTec2 (194,264), later studies reported mainly C4-oxidizing activity (187,192), as documented also in our batch of Cellic CTec2 (**Paper IV**). This shift could be explained by a rational change in the LPMO content of Cellic CTec2 after 2015. For instance, Vermaas et al. used molecular simulations to show that aldonic acids, the C1-oxidized glucose dimers, bound more tightly to cellobiose hydrolases than their C4-oxidized counterparts (252). In fact, D-glucono-1,5-lactone was shown to inhibit cellobiose hydrolases already in 1970 (265). Interestingly, cellobionic acid could relieve the inhibition of cellobiose hydrolases by cellobiose (266). Moreover, cellobionic acid can be cleaved to glucose and gluconic acid by β-glucosidases; whereas the C4-oxidized gemdiols cannot be cleaved, which may reduce final glucose yields. All in all, it is not easy to predict which LPMO and what level of LPMO activity is optimal to maximize saccharification yields, as highlighted also in a recent study by Østby et al. (94). In the work described in **Paper IV** we used *Ta*LPMO9A as Novozymes kindly provided us with this specific LPMO.

5.2.4 LPMO dosage

The dosing of LPMOs also must be considered. The LPMO dosage in products such as Cellic CTec2 is not known, but Müller et al. showed that substitution of 15% Celluclast with *Ta*LPMO9A resulted in the same final yields as those obtained with Cellic CTec2 (140). Two opposing approaches exist with respect to dosage: “top up” the cocktail with a specific enzyme, while leaving all other enzymes in the same amounts and ratios, or replace part of the cocktail and lower the final enzyme loading. Both present advantages and disadvantages. In **Paper IV**, we chose to top up the cocktails by 20% (w/w) with LPMOs, primarily because of the highly recalcitrant substrates utilized in this work.

5.2.5 Dry matter and storage of substrates

To improve the cost-effectiveness of biorefineries, elevated dry matter loading (up to 30%) must be used. Cannella et al. showed that the efficiency of LPMOs in various processing methods (simultaneous saccharification fermentation vs. separate hydrolysis and fermentation) varied according to dry matter loading (194,267). Finally, some studies point to the storage of substrates as another important factor to consider in LPMO applications, because redox and decarboxylating side reactions may interfere with subsequent LPMO activity during saccharification (268,269).

5.3 Role of AA9 LPMOs in saccharification of mildly pretreated spruce

Softwood is regarded as the “worst case scenario feedstock” (270). In 2015, only two pretreatments were known to achieve high cellulose conversion from softwood: steam pretreatment with SO_2 and organosolv pretreatment. The recalcitrance of steam-pretreated softwood has been associated with unspecific binding of enzymes to lignin and cellulose coverage by lignin, thus diminishing cellulase accessibility to the substrate (271). As explained above in subchapter 5.1, if the pretreatment is mild, the substrates contain hemicelluloses that also cover the cellulose.

Studies assessing the application of LPMO9s for softwood saccharification are scarce, and prior to **Paper IV**, the direct role of LPMO9s on mildly pretreated softwood was unknown. In most studies related to softwood saccharification and LPMO9s, the substrate had been harshly pretreated, and devoid of lignin and/or hemicellulose (140,192,216,272). For instance, the work by Hu et al. showed that LPMO9 addition increased hydrolysis mostly in corn stover (up to 25%), followed by hardwood (up to 20%) and then pine (up to 15%) (191). Chylenski et al. noted that pine was hydrolyzed more easily than spruce when using Cellic CTec3, with glucan conversion after 48 h amounting to 84.3% and 73.7%, respectively (192).

With the work in **Paper IV**, we addressed three main research questions: 1) could LPMO9s boost saccharification of mildly pretreated spruce, 2) by which mechanism, and 3) under what reaction conditions? We used three spruce substrates subjected

to increasing severity of pretreatment by steam explosion: I ($STEX_{210°C/auto}$), II ($STEX_{210°C/HAc}$), and III ($STEX_{210°C/SO2}$). Substrate III was pulp-like and served as a model of a less-recalcitrant substrate. Substrate I contained 6% hemicellulose (w/w dry mass), substrate II had 3% (w/w dry mass), and substrate III had none. Note that during pretreatment, substrates I and II lost the arabinose substitutions in arabinoglucuronoxylan and galactose substitutions in galactoglucomannan (247).

The work in **Paper IV** contributes to the LPMO field by reporting the effect of LPMO9s on saccharification of mildly pretreated softwood, one of the most recalcitrant lignocellulosic substrates. The Celluclast + Novozym 188 cocktail resulted in the highest saccharification yields on all three substrates, with 6% (I), 11% (II), and 66% (III) of glucose released (w/w glucan). Surprisingly, the LPMO9-containing Cellic CTec2 cocktail yielded lower glucose release, which made us wonder about ways of enhancing LPMO activity in our reaction setting.

Increasing aeration, which augmented the concentration of the co-substrate(s) O_2 and/or H_2O_2, did not improve saccharification, and neither did supplementation with ascorbic acid as an external electron donor (**Paper IV**). Similarly, Hu et al. noted that 10 mM gallic acid supplementation of Celluclast + *Ta*LPMO9A did not increase saccharification yields on any of their tested substrates (191). Nonetheless, **Paper IV** shows that lignin or its derivatives could, regardless of the pretreatment severity, be used as LPMO reductants in mildly pretreated softwoods, which might facilitate their saccharification at a commercial scale. It is likely that the substrates used in **Paper IV** release sufficient H_2O_2, because lignin may provide not only reductants but also H_2O_2 (195).

In the work described in **Paper IV**, supplementation of the benchmark cellulase cocktail Celluclast + Novozym 188 with *Ta*LPMO9A promoted glucose release (up to 1.6-fold) in all substrates. The effect was most pronounced at the end of the reactions, after 50 h (panels A, D and G in Figure 21), suggesting that LPMOs might degrade the most recalcitrant, leftover part of the substrates or that it takes time for other enzymes in the cocktails to utilize the LPMO-created nicks on recalcitrant cellulose. Wide-angle X-ray scattering revealed that LPMO addition significantly decreased the crystallinity of cellulose in substrates II and III. Curiously, LPMO remained active for a long time (at least up to 48 h) on all substrates (**Paper IV**), highlighting suitable reaction conditions where little or no (auto)inactivation took place. Furthermore, the LPMO used in this study likely possessed the protective methylation on the first histidine as well.

Interestingly, we noticed an increase in xylose release (up to 1.5-fold) in xylan-containing substrates I and II upon supplemented with *Ta*LPMO9A (panels B and E in Figure 21). This was surprising, as we expected addition of previously-known cellulose-active *Ta*LPMO9A to affect mainly glucose release.

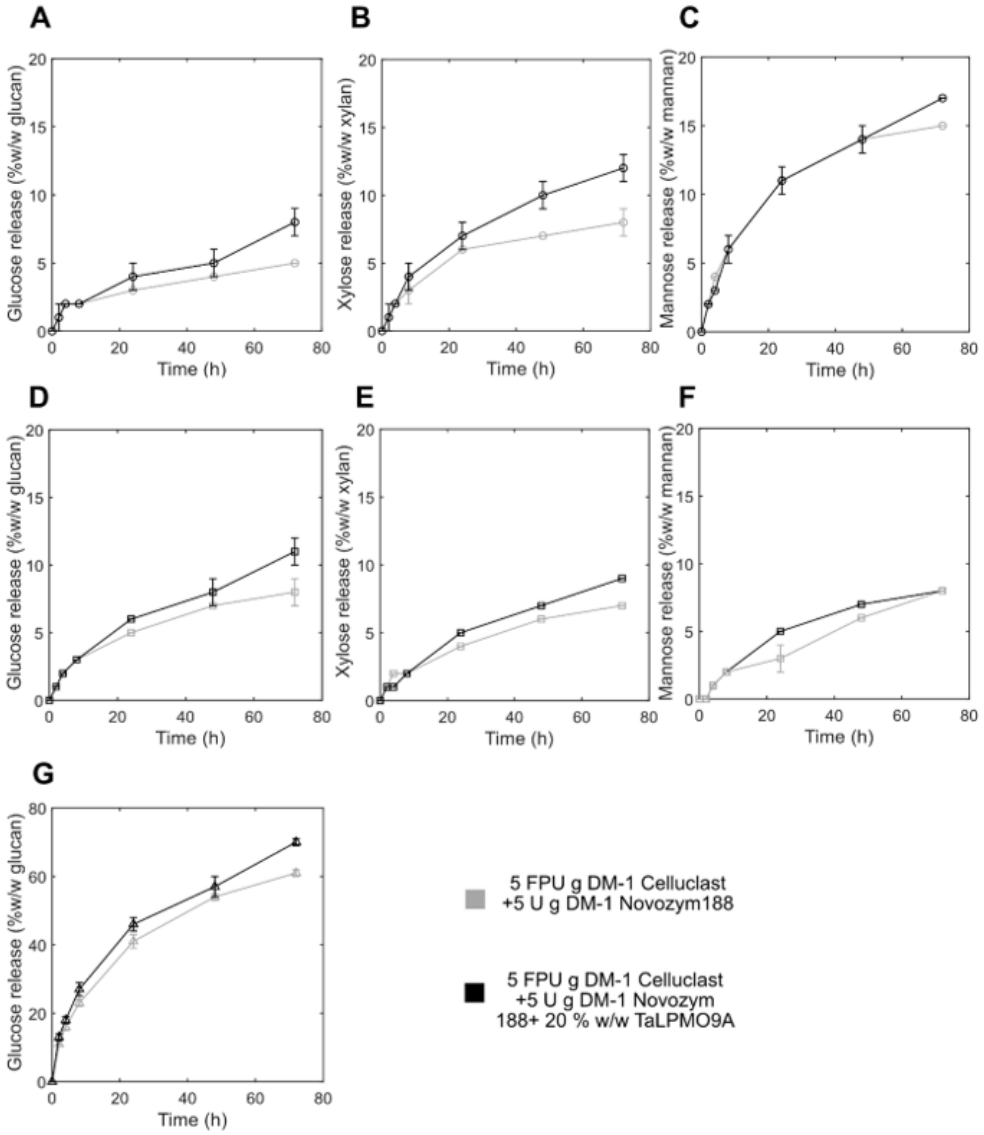


Figure 21. Time-course analysis of glucose (A, D and G), xylose (B and E) and mannose (C and F) release on substrate I (upper row), II (middle row), and III (bottom row) tested in Paper IV. *Grey plotted lines denote reactions without TaLPMO9A; whereas black plotted lines denote reactions with TaLPMO9A. The figure was derived from Paper IV.*

As shown in **Paper II** and other studies discussed in this thesis, several LPMO9s exhibit activity on xylan. Moreover, **Paper III** showed that use of debranched xylan (in substrates I and II arabinose had been removed during pretreatment) further enhanced the xylanolytic activity of LPMO9s. Considering that many LPMO9s may have xylanolytic activity on debranched xylan (combined with PASC) prompted me to test *Ta*LPMO9A activity on debranched spruce arabinoglucuronoxylan (**Paper III**) as well. As shown by HPAEC-PAD chromatograms (Figure 22), LPMO9 was active on debranched spruce arabinoglucuronoxylan complexed with PASC. MALDI-TOF MS confirmed the results from HPAEC-PAD as reductant dependent oxidized xylan-derived products were detected (Figure 23).

Nevertheless, caution is warranted when interpreting the results of *Ta*LPMO9A activity on xylan for several reasons. First, xylan content in substrates I and II was only 2% (w/w). Second, background enzyme activity, likely a xylanase from the *Ta*LPMO9A production host, was detected in the reductant-free control (Figure 22, peaks at 6.5 and 8.6 min in the second uppermost chromatogram). This may indicate minor xylanase background in our *Ta*LPMO9A batch that could have contributed to the increased xylose release apparent in Figure 21. Unfortunately, the peaks indicating possible contamination have not been identified yet, and thus the possible contaminating enzyme neither. Moreover, according to MALDI-TOF MS results (Figure 23), the oxidative regioselectivity of *Ta*LPMO9A seems to have shifted towards C1-oxidization on xylan as opposed to C4-oxidization on cellulose. The oxidized xylooligosaccharide peaks were very weak in the MALDI-TOF spectrum, whereby 0.3% relative intensity was used as cut-off for labelling the peaks in Figure 23. Also, such xylanolytic ability of *Ta*LPMO9A does not agree with the previously hypothesized structural features associated with xylanolytic activity of LPMO9s in the study by Hegnar et al. (242).

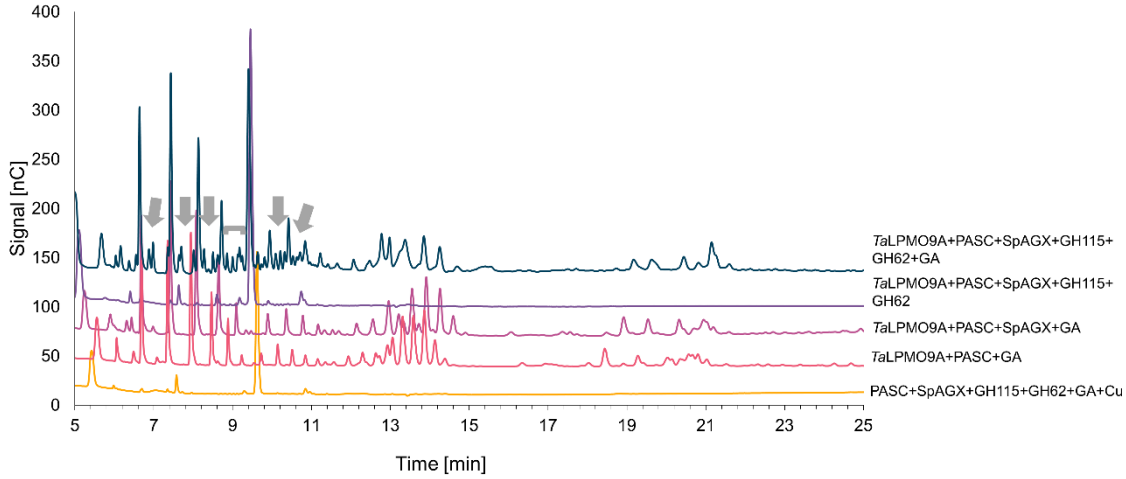
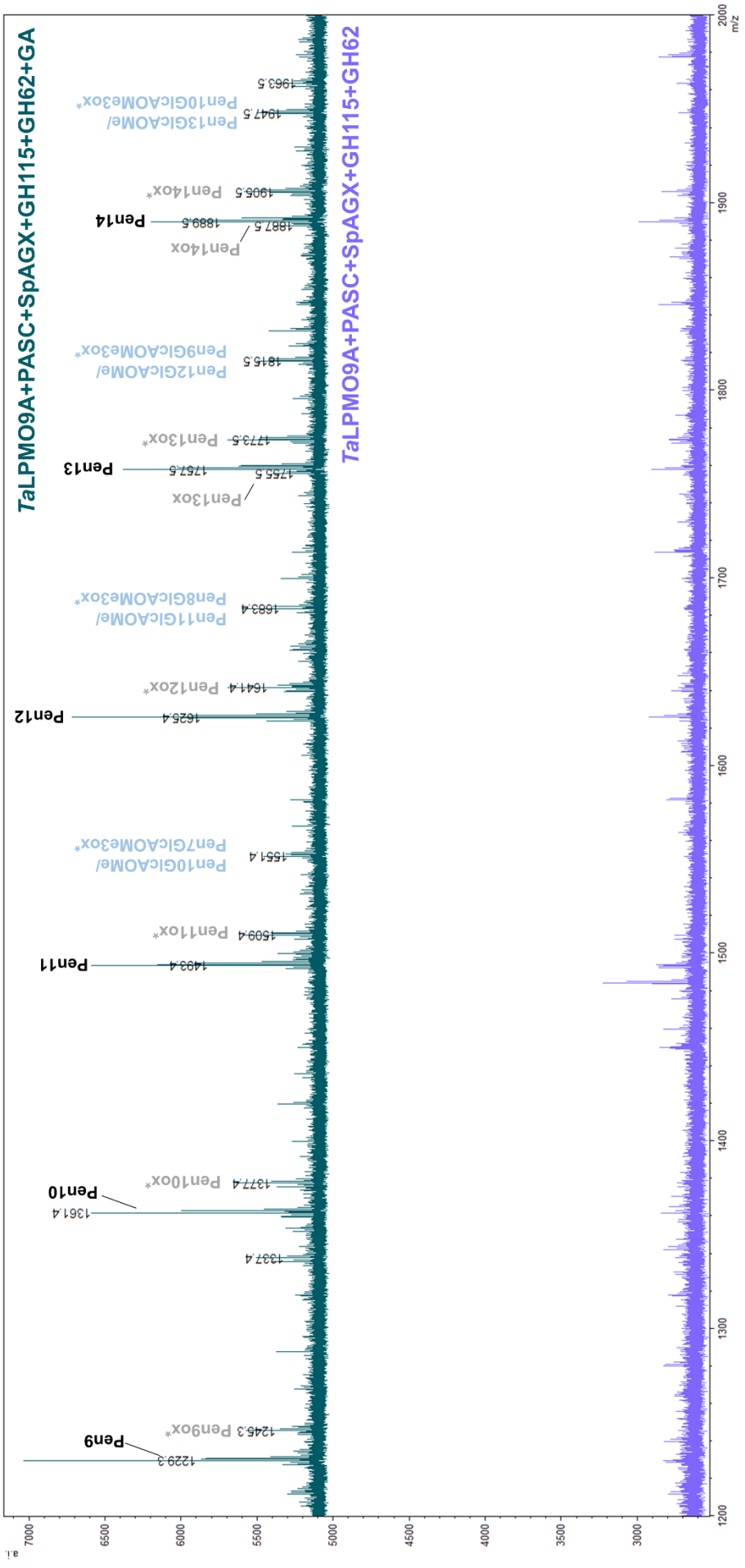


Figure 22. HPAEC-PAD chromatograms illustrating TaLPMO9A activity on PASC+spruce arabinoglucuronoxylan (SpAGX), in which SpAGX was debranched with GH62 and GH115 (uppermost green chromatogram)*. Only the green chromatogram shows xylan-derived products (labelled by arrows and a bracket). The reactions were carried out in duplicate in 100 µL at 40°C and 1000 rpm in 50 mM BisTris-HCl buffer (pH 6.5) for 16 h. The reactions contained 0.2% (w/v) PASC and 0.2% (w/v) SpAGX. The LPMO concentration was 1 µM and it was incubated with 0.9 eqm $CuSO_4$ for at least 30 min before starting the reactions. BoGH115 concentration was 100 nM and UmGH62 concentration was 15 µM. The reactions were started by adding 1 mM (final concentration) gallic acid (GA) except in reductant-free controls, where it was replaced with ultrapure water (purple chromatogram). In the LPMO-free control, LPMO was replaced with 0.9 µM $CuSO_4$ (yellow chromatogram). Prior to the HPAEC-PAD analysis, the samples were handled as described in Papers II and III.*

Still, the combination of results from three different analyses: saccharification, HPAEC-PAD, and MALDI-TOF MS point towards *Ta*LPMO9A having xylanolytic as well as cellulolytic activity, which could be relevant for biorefinery purposes. Interestingly, *Ta*LPMO9A was used earlier to supplement Celluclast + Novozym 188 when cleaving steam-exploded birch substrate containing 10% xylan (140). While only glucose release was quantified in that study, it would have been interesting to know if there was any effect on xylose.

LPMOs in future cocktails might benefit from dual cellulolytic/xylanolytic activities as this could lower total protein loadings. Moreover, it would be interesting to test if LPMO9s harboring xylanolytic activity are better than canonical xylanases at degrading recalcitrant xylans covering cellulose. Hu et al. showed that hemicellulose inhibited the production of CNFs, and xylanolytic AA9s might be beneficial in this case, too (273). (Hetero)xylans are underutilized in biorefineries compared to cellulose (111), and previously unknown xylanolytic activities of LPMO9s might provide LPMOs and xylans with novel applications.

Figure 23 (next page). MALDI-TOF analysis of TaLPMO9A activity on debranched spruce arabinoglucuronoxylan (SpAGX) complexed with PASC (in green), including the reductant-free control (purple). *GA, gallic acid. The two reactions shown are the same as in Figure 22 and were prepared in the same way. MALDI-TOF analysis was conducted as described in Paper II, except that 50% laser intensity was used. All marked peaks represent Na-adducts. PenX (in black) denotes native xylan-derived products where X is the degree of polymerization. PenXox* (in grey) denotes the hydrated forms of C1-oxidized xylan-derived products where X again marks the degree of polymerization. PenXox (in grey) denotes the C1-oxidized xylan products. Light blue denotes native singly methylglucuronated products and/or C1-oxidized hydrated xylan-derived products with triple methylglucuronic acid substitutions.*


TaLPMO9A+PASC+SpAGX+GH115+GH62+GA
TaLPMO9A+PASC+SpAGX+GH115+GH62
Pen9
Pen10
Pen11
Pen12
Pen13
Pen14
Pen9ox*
Pen10ox*
Pen11ox*
Pen12ox*
Pen13ox*
Pen14ox*
Pen10GlcAOMe/ Pen7GlcAOMe3ox*
Pen11GlcAOMe/ Pen8GlcAOMe3ox*
Pen12GlcAOMe/ Pen9GlcAOMe3ox*
Pen13GlcAOMe/ Pen10GlcAOMe3ox*
1229.3
1245.3
1337.4
1361.4
1377.4
1493.4
1509.4
1551.4
1625.4
1641.4
1683.4
1755.5
1757.5
1773.5
1815.5
1887.5
1889.5
1905.5
1947.5
1963.5
m/z
a.i.

5.4 AA9 LPMOs in the production of CNFs

The unique flat active site and powerful oxidative chemistry of LPMO9s (both explained in Chapter 4) has opened doors for developing novel applications for LPMO9s, in addition to their use in biomass saccharification. As explained in a review by Li et al., the smaller the scale of cellulose fibers, the greater the processing challenge (274). Thus, further research on processing methods is required to make nano-scale cellulosic products commercially available. LPMO9s could potentially contribute to cellulose modification at both nano- and micro scales, as I will explain below. The activity of LPMO9s on cellulose fibers has in some cases been studied to gain better understanding of overall LPMO activity on cellulose (233,254). Other studies on LPMO9s and cellulose fibers have been conducted to create and modify cellulosic materials.

CNFs are cellulosic nano-scale fibrils that have diameters in the range of 5–30 nm and lengths that can approach several microns (275). Current CNF production methods include chemical and mechanical steps, which are either toxic or have a high energy demand, making the process unsustainable (274). CNFs can also be prepared enzymatically by utilizing cellulases (276,277). CNFs are of interest as they can be derived from sustainable sources, such as wood and bacteria. Additionally, CNFs have interesting properties, including light weight, high mechanical strength, and biocompatibility to name a few. One noteworthy example from the potential use of CNFs comes from Japan (from the Nano Cellulose Vehicle project funded by the Japanese Ministry of Environment) where researchers were able to reduce the weight of a car by 10% by integrating CNFs in the car parts, thereby leading to 6%–8% lower fuel consumption as reviewed in (274).

Lately, LPMO9s have been used as a sustainable alternative for the production and chemical modification of CNFs. For instance, oxidized CNFs from delignified softwood fibers were produced using two *Nc*LPMO9s (18). In another case, CNFs were produced from kraft pulp by combining LPMO9s with other enzymes and this yielded CNFs with carboxyl content up to 100 mmol/kg (60% increase) (273). Han and colleagues demonstrated that a combination of enzymes including LPMO9s yielded CNFs from mechanically-treated pulp with high lignin content (278). Finally, Marjamaa et al. used *Tr*LPMO9s to oxidize fibers obtained from softwood kraft pulp (279).

5.5 AA9 LPMOs for the modification of sulfated CNCs

CNCs comprise the crystalline parts that remain after cellulose fibers are treated with mineral acids. CNCs are rod-shaped and can be derived from tunicates, bacterial cellulose, cotton, wood, wheat straw, and ramie (280). The main differences between CNFs and CNCs are dimensions and crystallinity. CNCs are generally highly crystalline, with length below 500 nm (281). Their sustainable provenance, as well as mechanical and chemical characteristics make CNCs an appealing material. Commercially available CNCs are produced using sulfuric acid and contain sulfate half-ester groups on their surface (Figure 6b). These charged

groups improve the colloidal dispersibility of CNCs. CNCs are promising building blocks for example in biomedical engineering, drug and gene delivery, wastewater treatment, and protein scaffolds (280,281).

CNCs can be chemically modified to provide novel properties for subsequent coupling chemistry. The three available hydroxyl groups per anhydroglucose can, for instance, be used for oxidative functionalization (282). The most common chemical oxidations on CNCs are TEMPO-mediated carboxylation at C6 (283) or carbonylation of C2 and C3 by periodate oxidation (Figure 6c) (48). As none of these methods are sustainable, LPMOs could represent a possible alternative. As described in subchapter 4.2, the C1-specifc LPMO reaction products are lactones in equilibrium with aldonic acids. Glucolactone with degree of polymerization 2 is in pH-dependent equilibrium with cellobionic acid: the former prevails at acidic pH; whereas the latter prevails at more basic conditions (284). The pKa of carboxylates on cellulose is around 4 (285). To the best of my knowledge, the equilibrium between the C4-oxidized LPMO reaction products is less well known. The carboxyl groups produced by LPMOs are interesting for CNC functionalization because of their possible charge and subsequent use of the carboxyls as “reactive handles” (286). The oxidation of C6 in cellulose by LPMO9s or other enzymes would also be very interesting, as technically it would likely not lead to chain cleavage as C1 and C4 oxidations do, but this possibility has not been proven yet. C6 oxidation by LPMOs is part of double C1/C6 or C4/C6 oxidation as explained in subchapter 4.2 (157,209–211); whereas single C6 oxidation by LPMOs may need to be explored in the future. One intriguing option is the discovery or engineering of non-specific galactose oxidases, which could introduce C6 oxidations onto cellulose. Galactose oxidases have already shown great potential for the oxidation of galactose-containing hemicelluloses in biomimetic aerogels (287).

In recent years, production of CNCs by LPMOs has been demonstrated, and a few studies have illustrated the functionalization of CNCs by LPMOs. In the work presented in **Paper V,** we aimed at performing a systematic study on the effect of sulfate half-ester groups on CNC functionalization by LPMO9s that so far have been lacking. Karnaouri and colleagues investigated whether the C1/C4-oxidizing *Mt*LPMO9H could be employed to produce and modify CNCs from tunicate-derived cellulose (288). Even though the exact sulfur content was not reported for all substrates, X-ray photoelectron spectroscopy indicated that *Mt*LPMO9H could indeed oxidize sulfated CNCs. Crucially, the crystallinity index and crystal dimensions decreased only minimally after LPMO treatment, and the crystals did not lose their shape (288). Muraleedharan et al. used organosolv-pretreated birch as a starting material in a similar study (289). C1-oxidizing *Pc*LPMO9D rather than C1/C4-oxidizing *Mt*LPMO9H was able to oxidize the insoluble parts of sulfate-containing CNCs, even though *Mt*LPMO9 altered the dimensions of the crystals. The authors hypothesized that net-neutral carboxylation took place, whereby the amount of carboxyl groups removed (originally present in the substrate) was equal to the amount of carboxyl groups added by LPMOs.

Solhi and colleagues tested the ability of a bacterial AA10 LPMO to oxidize the surface and release soluble products on numerous CNFs and CNCs with different chemical modifications and with various LPMO loadings. They used an AA10 and sulfated CNCs, which can be compared to HCl-produced CNCs in their study (the latter resemble most the desulfated CNCs used in **Paper V**). However, in the work by Solhi et al., the least amount of soluble products were released on sulfated CNCs, and the increase in carboxyl group on sulfated CNCs was relatively low also, only 2% (286).

Magri et al. tested seven LPMO9s with differing oxidative regioselectivity on sulfated CNCs and PASC. Based on the analysis of soluble products, the degree of oxidation on sulfated CNCs appeared to be both substrate- and LPMO-dependent (290). Unfortunately, the carboxyl content on the insoluble part of the CNCs was not analyzed in this study. A remarkable effect of dry matter loading was noted, with some LPMOs exhibiting activity on sulfated CNCs only at high dry mass values (6.5% w/w). Additionally, the crystallinity index decreased after treatment with some LPMOs. Koskela and colleagues achieved elevated production and oxidation of CNCs by LPMO9s, but their starting cellulose material did not contain sulfate groups and had much larger dimensions owing to a microcrystalline structure (19).

In summary, the direct role of sulfate half-ester groups on LPMO9 oxidation of CNCs remains poorly explored. However, the relevance of sulfate groups should not be discarded, as such half-esters block cellulases in Celluclast (291) and also hinder the Na-periodate reaction (48). To bridge this gap, we studied how the C1-oxidizing and CBM-containing *Tt*LPMO9G characterized in **Paper II** and **III** was affected by such sulfate half-ester groups, and whether it could carboxylate sulfated CNCs for subsequent crosslinking (**Paper V**). For the research in **Paper V**, we analyzed both the soluble and solid fraction resulting from LPMO catalysis (Figure 24).

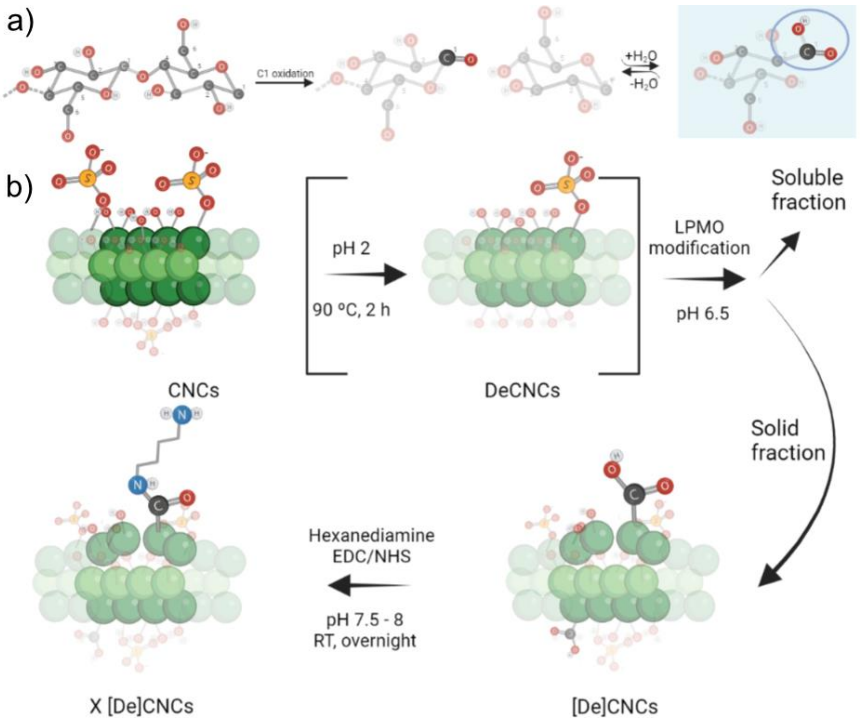


Figure 24. Workflow of Paper V. *Desulfated CNCs (deCNCs) were generated by applying first acid-catalyzed desulfation (pH 2, 90°C, 2 h), followed by LPMO modification (pH 6.5, RT, 6 h) of both sulfated and desulfated CNCs. The solid fractions from both reactions were then used for EDC/NHS-mediated crosslinking with hexanediamine (pH 7.5–8.0, room temperature, overnight) to prove LPMO oxidation. Note that direct analysis of soluble products from the LPMO reactions analyzed in Figure 25 and 26 was not possible. The reactions for soluble product analysis were prepared separately at a smaller scale and analyzed with HPAEC-PAD (Paper V). The figure was retrieved from Paper V.*

*Tt*LPMO9G was chosen as the catalyst for the work in **Paper V** due to its C1-specific oxidative regioselectivity and its strong activity on various cellulosic substrates (**Paper II**). Furthermore, this LPMO could be produced in high quantities as the reactions with CNCs had to be scaled up to 100 mL for subsequent analyses. An attempt to produce *Tt*LPMO9G in a bioreactor using fed-batch mode in a volume of 1 L was made, but the yield was very small. Hence, further optimization is needed to produce this LPMO using bioreactors.

In the work described in **Paper V**, we assessed whether *Tt*LPMO9G was active on both sulfated and desulfated CNCs, whether it could modify the insoluble parts of sulfated and desulfated CNCs without notable changes to the structure and colloidal stability of CNCs, and whether the carboxyl groups produced by the LPMO could be exploited for subsequent crosslinking. Analysis of soluble oxidized products using HPAEC-PAD showed that *Tt*LPMO9G was indeed active on both substrates. Semi-quantification of the released products indicated that *Tt*LPMO9G released 27% more soluble oxidized products from desulfated CNCs compared to non-sulfated CNCs. Analysis of the insoluble reaction products by conductometric titration (Figure 25) and attenuated total reflectance Fourier transform infrared spectroscopy (Figure 26) revealed no carboxyl groups on desulfated CNCs after LPMO treatment.

Instead, the carboxyl groups on sulfated CNCs had increased by 10% (from 0.20 to 0.22 mmol/g) after 6 h of treatment with *Tt*LPMO9G (Figure 25). Such results contradicted our initial hypothesis, as we assumed that *Tt*LPMO9G oxidized preferably desulfated CNCs owing to less steric hindrance. Notably, the work in **Paper V** showed that the sulfate half-ester groups promoted the carboxylation of CNCs by *Tt*LPMO9G as they likely prevented localized cuts, which would release more soluble cellooligosaccharides. As proposed by Muraleedharan et al. (see above in this subchapter), it is also possible that the activity of *Tt*LPMO9G on desulfated CNCs was net-neutral.

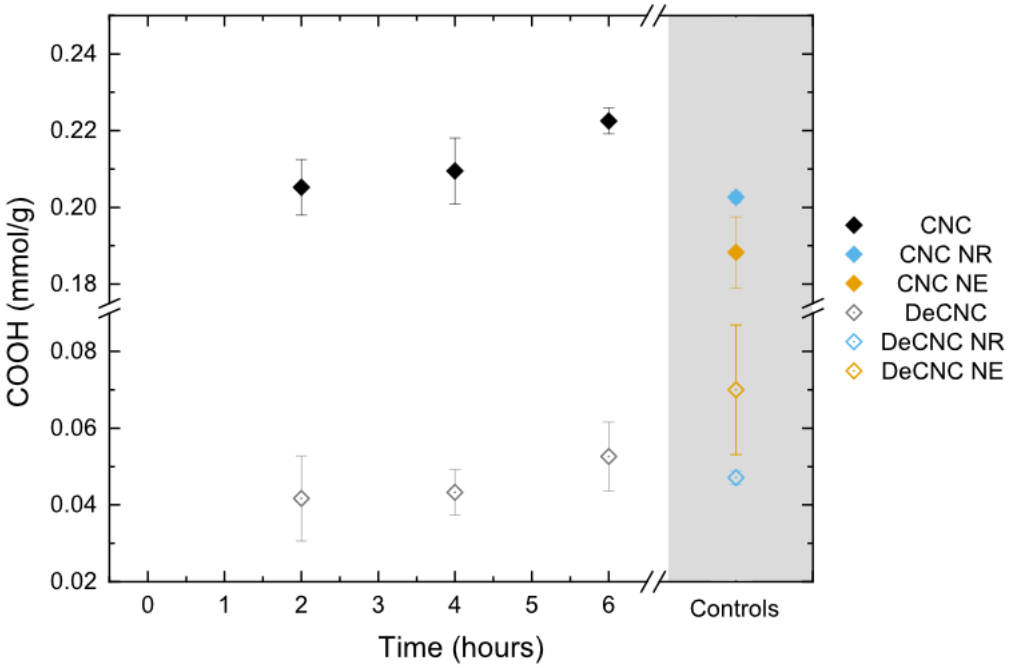


Figure 25. Carboxyl group content of TtLPMO9G-treated CNCs and desulfated CNCs measured by conductometric titration. *CNC indicates sulfated CNCs and DeCNC indicates desulfated CNCs. NE refers to enzyme-free controls and NR to reductant-free controls. Error bars denote the standard deviation of at least triplicate independent repetitions. The figure was retrieved from Paper V.*

With the work in **Paper V** we showed that neither colloidal stability nor CNC dimensions were significantly affected by CNC treatment with *Tt*LPMO9G. Additionally, as shown in Figure 26, the 10% increase in carboxyl groups achieved by LPMO was sufficient to crosslink CNCs using EDC/NHS and hexadiamine. The spectra in Figure 26 indicate a reduction of the peak denoting *Tt*LPMO9G-introduced carboxyl groups (1740 cm^{-1}) on sulfated CNCs after overnight crosslinking, with a new amide bond (1570 cm^{-1}) appearing instead as a result of successful crosslinking/coupling chemistry.

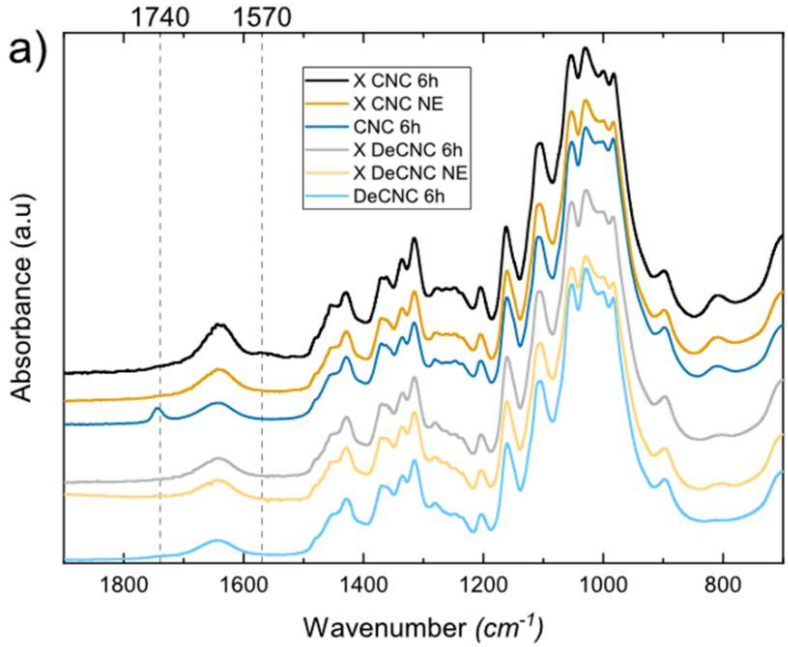


Figure 26. Attenuated total reflectance Fourier transform infrared spectroscopy spectra from 1900 to 700 cm^{-1} illustrating the crosslinking (marked as X in the legend) of LPMO-modified CNCs using EDC/NHS and hexadiamine*. The peak marked at 1740 cm^{-1} corresponds to carboxyl groups introduced by TtLPMO9G and the peak at 1570 cm^{-1} corresponds to secondary amides formed during the crosslinking. CNC indicates sulfated CNCs and DeCNC indicates desulfated CNCs, while NE denotes enzyme-free controls. 6h indicates the length of the LPMO-mediated modification of the (De)CNCs. The figure was retrieved from Paper V.*

In summary, the work in **Paper V** offers direct evidence that sulfate half-ester groups favor the functionalization of CNCs by *Tt*LPMO9G. In the future, it would be highly interesting to test different reaction conditions to further augment the carboxyl content, which was only 0.22 mmol/g after 6 h of *Tt*LPMO9G treatment in the study shown in **Paper V**. Testing the effect of the CBM on the same catalytic LPMO9 domain, temperature, pH, reaction duration, reductants, and controlled H_2O_2 feeding could further improve the carboxylation of CNCs by *Tt*LPMO9G. It is likely that carboxylation by the LPMO is tunable by varying these parameters, and this may lead to CNCs with novel characteristics. **Paper V** also demonstrates a proof-of-concept crosslinking mechanism for sulfated CNCs, which can be exploited in the future to produce bio-based materials. Distinct catalytic LPMO9 domains likely have different functionalization capacities, which cannot be generalized based solely on **Paper V** and published data. A comparison of soluble vs. insoluble products in **Paper V** indicates that a higher concentration of soluble oxidized products does not necessarily coincide with more surface oxidations, and *vice versa*.

6. Conclusions

In summary, the work described in my thesis illustrates the process of discovering, functionally characterizing, and applying LPMO9s in a biorefinery context.

Using genome and transcriptome analyses of the fungal strain *T. terrestris* LPH172, I showed that this strain has a well-equipped enzymatic machinery to degrade lignocellulosic biomass (**Paper I**), like the previously sequenced and analysed NRRL 8126 strain of the same species (131). Detailed analysis of the genes expressed during growth on Avicel, rice straw and beechwood xylan showed that different CAZyme-encoding genes were upregulated depending on the substrate, suggesting a detailed regulation of CAZyme-encoding genes in response to growth on different lignocellulosic substrates, and pointing to the mechanisms *T. terrestris* LPH172 uses to break down lignocellulose. The gene expression analysis also showed a nuanced co-upregulation of putative synergetic enzyme encoding LPMO9 genes together with other AA family CAZyme genes; and, for example, xylanase genes appeared to act in concert with genes encoding xylan debranching enzymes. The high number of upregulated and highly expressed LPMO9 genes on Avicel, rice straw and beechwood xylan showed that LPMO9s are of high importance for lignocellulose degradation in some filamentous fungi, including *T. terrestris* LPH172. The identified*Tt*LPMO9-encoding genes were the basis for subsequent functional characterisation (**Paper II** and **Paper III**) and application (**Paper V**) of LPMO9s.

The multiplicity of LPMO9 encoding genes in *T. terrestris* could be explained by the functional differences exhibited by the individual enzymes (**Paper II**). The work presented in **Paper II** supports the hypothesis that filamentous fungi harbour plentiful LPMO9 genes as the LPMO9s they encode attack different polysaccharides in plant cell walls, or degrade same polysaccharides with different specificity (e.g. the sensitivity to TXG substitutions). The functional characterization in **Paper II** revealed novel LPMO9 activities on acetylated birch glucuronoxylan and spruce arabinoglucuronoxylan (when combined with cellulose) for the first time.

Interestingly, none of the tested *Tt*LPMO9s showed activity on highly substituted arabinoxylan from wheat (**Paper II**) even if some of them were active on other tested xylan substrates. By utilizing arabinoxylans with different substitution levels and by enzymatically debranching xylans, I showed that xylanolytic capabilities of LPMO9s can be increased (e.g. *Tt*LPMO9G activity on spruce arabinoglucuronoxylan) and previously unknown activities revealed (e.g. *Tt*LPMO9E activity on wheat arabinoxylan and spruce arabinoglucuronoxylan) (**Paper III**). This effect is likely due to improved adsorption onto cellulose by debranched xylan, which makes the xylan conformation more accessible to LPMO9s. Improved LPMO action upon debranching can also be due to the removal of steric hindrance from the LPMO. The importance of cellulose as a co-substrate for interaction with the xylans was also corroborated both in **Paper II** and **Paper III**, as xylanolytic activities of LPMO9s were only detected in xylan-cellulose mixtures.

My work in **Paper III** shows that some LPMO9s have significant xylanolytic activity which will likely play an important role in different LPMO applications in the future, from enzymatic saccharification to functionalization of xylan-based materials. The xylanolytic activities of LPMO9s demonstrated in **Paper III** are also important in mapping the native functions of LPMO9s in plant cell wall degradation. The functional characterization in **Paper II** and **Paper III** underlines the importance of using substrates in their most native-like environment and conformation, as some functionalities will be overlooked otherwise when functionally characterizing LPMOs. LPMO9s with dual cellulolytic/xylanolytic activity have the potential to reduce protein loadings in enzymatic saccharification, and thus also reduce the cost of this biorefinery process. Moreover, it is possible that LPMO9s compared to canonical xylanases are better equipped to degrade recalcitrant cellulose-adsorbed xylans in lignocellulosic biomass.

In the work shown in **Paper IV,** me and my co-authors investigated if LPMO9s can contribute to increasing the sugar yields of enzymatic saccharification of highly recalcitrant mildly pre-treated spruce substrates. We also studied the possible underlying mechanisms of increased sugar release, along with optimizing the reaction conditions. The work in **Paper IV** substantiated the notion that enzymatic cocktails need case-by-case development depending on the feedstock and the pretreatment used as we detected lower saccharification yields with a more advanced cocktail Cellic CTec2 than with the benchmark cocktail Celluclast + Novozym 188 under our specific experimental conditions. Nonetheless, direct LPMO9 supplementation of Celluclast + Novozym 188 with *Ta*LPMO9A was beneficial for the saccharification of mildly pretreated spruce substrates as both glucose and xylose yields were increased. Interestingly, the increased xylose release may be attributed to *Ta*LPMO9's capability to also degrade cellulose-bound arabinoglucuronoxylan as I showed in experiments related to **Paper IV** in subchapter 5.3. In the work demonstrated in **Paper IV** we showed by wide-angle X-ray scattering analysis that *Ta*LPMO9A decreased the crystallinity of the substrates, and that the lignin present in all of the used substrates with different pretreatment severities served as a sufficient reducing agent for the LPMO. The work in **Paper IV** therefore supports the notion that LPMO9s in lignocellulolytic cocktails can degrade recalcitrant crystalline cellulose and thereby enhance the activity of other cellulases.

In my work in **Paper V**, I used *Tt*LPMO9G (characterized in **Paper II**) to add carboxylation functionalities on CNCs for proof-of-concept crosslinking. *Tt*LPMO9G released more soluble oxidized cellooligosaccharides from desulfated CNCs, but the LPMO-catalyzed carboxyl groups were only retained on the sulfated CNCs. My work in **Paper V** shows that chemical groups covering substrate surfaces in some cases can be beneficial for the use of LPMOs, depending on the required activity from LPMOs. The sulfate half ester groups hindered the LPMO to such an extent that the catalyzed carboxyl groups remained in the solid fraction after the reaction, as targeted in the study described in **Paper V**, instead of unfavoured excessive surface degradation into small soluble products. The ratio of solid part oxidation over soluble product release likely depends on the specific LPMO applied and the reaction conditions used, which opens possibilities to tune CNC oxidation by LPMOs. The study described in **Paper V** shows that it is possible to functionalize

sulfated CNCs with LPMOs to the extent that the LPMO-added functional groups can be used for subsequent crosslinking chemistry.

LPMOs have an untapped potential for being applied in a biorefinery context. The work in **Paper V** demonstrates how LPMO9s can be applied for (sulfated) nanocellulose functionalization for producing sustainable bio-based materials. The work in **Paper IV** brings mildly pretreated softwood as highly recalcitrant substrate one step closer to being used as a feedstock in future biorefineries.

A basis for application of LPMOs is a functional characterization of their action. By applying a broad range of native-like substrates and combining different analytical methods, I have substantially accelerated the functional understanding of LPMO9s. The previously unknown xylanolytic activities of both *Ta*LPMO9A (**Paper IV**) and *Tt*LPMO9E (**Paper III**) show that even well-studied LPMOs may have functionalities that the scientific community is unaware of, and likely more new functionalities will be found in the future. The demonstrated xylanolytic activities of LPMO9s will open the possibility to use them in production of hemicellulose-based materials, and this will hopefully contribute to finding new applications for underutilized hemicelluloses. It is also expected that some of the tested LPMO9s (e.g., *Tt*LPMO9U) have novel substrate preferences as they did not show high (or any) activity on the tested polysaccharides.

7. Future perspectives

My work expands our understanding of AA9 LPMOs and sheds light on the question of why fungi harbour so many AA9-encoding genes. Filamentous fungi are extremely diverse and remain enigmatic. It is plausible that fungi use different mechanisms for polysaccharide degradation, analogous to those employed by white and brown rot fungi to convert lignin. From a fundamental research perspective, it would be interesting to study further the different lignocellulose-degrading mechanisms employed by fungi. In particular, I would like to elucidate further which enzymes act in synergy with LPMOs in nature, and how LPMO expression is regulated in filamentous fungi.

I expect that future enzyme discovery will be improved by more efficient cloning and protein production systems, as predicted by ongoing advances in molecular, genetic, bioprocess, and automation tools. It would be exciting to find out why some LPMO9s can be produced in a specific host, while others cannot. Solving expression issues would facilitate the characterization of complete sets of LPMO9s in *T. terrestris* LPH172 and other organisms, and further amplify our knowledge on the multiplicity of these genes. This might also contribute to our understanding of LPMOs as virulence factors or lead to the discovery of completely novel LPMO functions.

An intriguing candidate for future studies is *Tt*LPMO9U, which had no activity on any tested polysaccharide. The same can be said of other less active *Tt*LPMO9s, whose true activity may arise by varying conditions and substrates. Other missing pieces of the puzzle are all the insoluble products resulting from LPMO activity and the remaining insoluble fraction of the substrate, whose oxidation pattern may offer new insights. I am also excited to see future work on the possible role of LPMOs on lignin modification and/or degradation, as it has both fundamental and industrial importance.

Additionally, I would like to study in more detail if and how the synergy between debranching glycoside hydrolases and LPMOs is relevant for lignocellulose degradation. Why did we not see the same synergy on Avicel? What happens using natural substrates? It would be interesting to test if LPMOs are sterically hindered by the remaining substitutions on cellulose-bound xylan, or if the increase in LPMO activity upon debranching only relates to improved/changed cellulose binding that gives xylan an LPMO-susceptible conformation. Of note, unraveling the structural features that govern xylanolytic LPMO9 activity are also of high importance. It is possible that xylan-active LPMOs can be exploited to produce interesting biological materials just like their cellulolytic activities have been used in applications on cellulose.

Regarding mildly pretreated spruce, I would like to further study the effect of LPMO addition at different timepoints and understand the role of lignin in these substrates in more detail. Additionally, saccharification yields should be increased to more industrially acceptable levels by, for example, evaluating the role of H_2O_2 feeding, catalase supplementation, higher dry mass concentrations, and different

temperatures, possibly using a design of experiments approach. The present work on CNCs could pave the way for actual applications of sulfated and LPMO-oxidized CNCs in a biomedical context. To this end, it would be important to further optimize reaction conditions for the addition of carboxylic acid groups on sulfated CNCs by LPMO9s.

Finally, I would like to explore, whether the new enzyme functionalities described above can bring down the cost of lignocellulolytic enzyme cocktails and thus enable economically viable production of biofuels and biochemicals. I hope that the knowledge obtained here from degrading lignocellulose will also teach us how to degrade other recalcitrant materials such as plastics. It is possible that even LPMOs will be used one day for this purpose, thanks to their oxidative power.

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