THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Towards a tailor-made enzyme cocktail for saccharification of mildly steam pretreated Norway spruce

Biomass structure guides enzyme supplementation

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Department of Life Sciences
CHALMERS UNIVERSITY OF TECHNOLOGY
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Cover: Always smile, even if your smile is sad, because sadder than a sad smile is the sadness of not knowing how to smile.
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Be always like the sea,

than breaking up against cliffs it

finds always the force to try again.

-Jim Morrison

Preface

This dissertation partially fulfils the requirements for obtaining the degree of Doctor of Philosophy at the Department of Life Sciences, Chalmers University of Technology. The work was supported by the Swedish Energy Agency (2019-021473) through a grant awarded to Prof. Lisbeth Olsson. The PhD studies were carried out between June 2020 and December 2024 under the supervision of Prof. Lisbeth Olsson and co-supervision of Dr. Vera Novy, with Prof. Carl Johan Franzén as examiner.

Most of the work in this thesis was carried out at the Division of Industrial Biotechnology, Department of Life Sciences, Chalmers University of Technology. Pretreatment of the materials and NMR analysis (Paper I) were performed at Lund University by Dr. Basel Al-Rudainy. SAXS/WAXS (Papers I, II, III) analyses were performed by Dr. Polina Naidjonoka, Division of Materials Physics, Department of Physics, Chalmers University of Technology. SEM imaging (Paper II) was performed by Dr. Maria Brollo, Department of Physics, Chalmers University of Technology. FTIR measurements (Paper V) were performed by Åke Henrik-Klemens, Division of Applied Chemistry, Department of Chemistry and Chemical Engineering, Chalmers University of Technology. Proteomic data analysis (Paper IV) was performed by Romanos Siaperas from the Industrial Biotechnology & Biocatalysis Group, National Technical University of Athens.

Fabio Caputo,
December 2024

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Abstract

The use of spruce in biorefineries (refineries converting biomass into bioproducts) is limited due to its recalcitrance to enzymatic saccharification (process of breaking down polymers into monomers using enzymes). To overcome this issue, harsh steam pretreatment conditions can be applied, even though they result in yield losses caused by (hemi-)cellulose solubilization and lignin condensation. The aim of this thesis was to build the foundation for tailor-made cocktails for the enzymatic saccharification of mildly steam pretreated spruce, using structural knowledge of the material before and after enzymatic hydrolysis. Spruce was steam pretreated under different conditions ranging from low to high severities. Advanced analytical techniques revealed diverse hemicellulose content and structural characteristics following the different pretreatment procedures.

Based on the structural changes, a cellulolytic cocktail was supplemented with enzymes that targeted different structures in steam pretreated spruce. Lytic polysaccharide monooxygenases were supplemented to enhance the hydrolysis of highly ordered cellulose. Hemicellulases were supplemented to remove the hemicellulose shield and increase accessibility of enzymes to cellulose. Finally, the rearrangement of lignin in less severely steam-treated materials was tested by laccase supplementation. Changes in saccharification yields were detected in relation to supplementation strategy and led to unique alterations in the structural characteristics of the residual material. Enzymes secreted by the fungus *Thermothielavioides terrestris* grown on different steam pretreated materials were studied. Even minor changes in the structure of spruce affected composition of the fungal secretome, indicating that each steam pretreated substrate required a different enzyme ratio for effective degradation. The secreted enzymes were tested as a supplement to the cellulolytic cocktail, resulting in increased saccharification.

Overall, this thesis highlights the importance of combining structural knowledge before and after hydrolysis with understanding of enzymatic function. Together, they can drive the development of tailor-made cocktails for more efficient lignocellulose saccharification.

Keywords: softwood, steam explosion, pretreatment, enzymatic hydrolysis, LPMOs, laccase, filamentous fungi

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Caputo F.**, Al-Rudainy B., Naidjonoka P., Wallberg O., Olsson L., Novy V. Understanding the impact of steam pretreatment severity on cellulose ultrastructure, recalcitrance, and hydrolyzability of Norway spruce. Biomass conversion and biorefinery, 2022. DOI: 10. 1007/s13399-022-03405-0

II. Brollo M.E.F.*, **Caputo F.***, Naidjonoka P., Olsson L., Olsson E. Overall structural changes and cellulose ultrastructure after enzymatic hydrolysis of mildly steam pretreated Norway spruce. (manuscript under revision)

III. **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. Biotechnology for biofuels and bioproducts, 2023. DOI: 10.1186/s13068-023-02316-0

IV. **Caputo F.***, Siaperas R.*, Dias C., Nikolaivits E., Olsson L. Elucidating *Thermothielavioides terrestris* secretome changes for improved saccharification of mild steam-pretreated spruce. Biotechnology for biofuels and bioproducts, 2024. DOI: 10.1186/s13068-024-02569-3

V. **Caputo F.**, Henrik-Klemens Å., Hilden K., Horn J.S., Eijsink H.G.V., Olsson L. Insights into the combined action of laccase and LPMOs for steam-pretreated spruce saccharification. (short communication - manuscript)

*These authors contributed equally

Contribution Summary

I. I contributed to the conceptualization of the study. I acquired and analysed the data on enzymatic hydrolysis and carbohydrate quantification in biomass. I wrote and edited the manuscript together with the other authors.

II. I contributed to the conceptualization of the study. I planned the study and performed the experimental work together with Dr. M.E.F. Brollo. I analysed the data, interpreted the results, and wrote the manuscript. I edited the manuscript together with the other authors.

III. I contributed to the conceptualization of the study. I planned and performed the experiments, as well as analysed the data. I interpreted the results and wrote the manuscript together with Dr. M. Tölgo. I edited the manuscript together with the other authors.

IV. I conceived the study and planned the experiments together with Dr. E. Nikolaivits. Most of the experimental work was performed by C. Dias, whom I supervised. The bioinformatics analysis of proteomic data was performed by R. Siaperas. I analysed the data, interpreted the results, and wrote the manuscript together with R. Siaperas. I edited the manuscript together with the other authors.

V. I conceived the study, performed the experiments, and analysed the data. I interpreted the results and wrote the manuscript. I edited the manuscript together with the other authors.

Other related publications (not included in the thesis)

I. Torello Pianale, L., **Caputo, F.**, Olsson, L. Four ways of implementing robustness quantification in strain characterisation. Biotechnol Biofuels 16, 195 (2023). https://doi.org/10.1186/s13068-023-02445-6

II. Henrik-Klemens Å., **Caputo F.**, Ghaffari R., Westman G., Edlund U., Olsson L., Larsson A. The glass transition temperature of isolated native, residual, and technical lignin. Holzforschung, vol. 78, no. 4, 2024, pp. 216-230. https://doi.org/10.1515/hf-2023-0111

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Abbreviations

STEX	Steam pretreatment
CSF	Combined severity factor
HAc	Aceti acid
DM	Dry mass
S	Syringyl
G	Guaiacyl
H	p-Hydroxyphenyl
LCCs	Lignin carbohydrate complexes
SAXS	Small angle X-rays scattering
WAXS	Wide angle X-rays scattering
SEM	Scanning electron microscopy
HSQC 2D-NMR	Heteronuclear single quantum coherence 2 dimensional- nuclear magnetic resonance
HMBC 2D-NMR	Heteronuclear multiple bond correlation 2 dimensional- nuclear magnetic resonance
HPAEC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
FTIR	Fourier-transform infrared spectroscopy
ATR	Attenuated total reflectance
FPU	Filter paper unit
LPMOs	Lytic polysaccharide monooxygenases
CBH	cellobiohydrolases
CDH	cellobiose dehydrogenases
CBM	carbohydrate-binding module

Chapter 1. Introduction

Climate change requires switching from a linear economy to a circular one and policy makers are tasked with enabling this transition (Leong et al., 2021). A first step was the 2016 Paris agreement, signed by 195 countries (UNFCCC, 2016). In 2023, the Net Zero Emission by 2050 Scenario was released (IEA, 2023). It is "a normative scenario that presents a workflow for the global energy sector to achieve net zero CO_2 emissions by 2050, with advanced economies reaching net zero emissions in advance compared to emerging market and developing economies". Notably, the different measures discussed in this scenario include the use of lignocellulosic biomass in biorefineries.

1.1 Biorefinery concepts and showcase examples

According to the International Energy Agency, biorefinery is defined as the processing of renewable biomass feedstocks into marketable energy, chemical, and material products (de Jong et al., 2011). The aim of a biorefinery is to produce "platform chemical" intermediates, such as pentose (C5) and hexose (C6) carbohydrates, syngas, lignin, or pyrolytic liquids, starting from different types of feedstocks (i.e. agricultural/forestry crops and residues or municipal solid waste) (Asghar et al., 2022). The intermediates are valorised through a range of energy carriers, chemicals, materials, and food and feed constituents (**Figure 1.1**). Therefore, a key goal of a biorefinery is the valorisation of all fractions obtained from the starting biomass (Cherubini, 2010). There are many ways to fractionate biomass; they depend on the target feedstocks and products (Galbe & Wallberg, 2019).

The Borregaard wood biorefinery in Norway fractionates spruce into cellulose, hemicellulose, and lignin to produce chemicals and materials. Borregaard is an established supplier to manufacturers of various cellulose derivatives, including ethers, acetate, nitrocellulose, and fibrils (used as additives). The sugars from hemicellulose are utilised in the production of bioethanol. Lignin is either converted into lignin-based biopolymers or used in the production of biovanillin (https://www.Borregaard.com/). The Södra biorefinery in Sweden converts wood raw feedstock into building materials, pulps, and biofuels via a zero-waste approach. For instance, tall oil obtained from black liquor, which is a side product of the pulping process, is used as additive in many everyday products, including soap, glue, tape, facade and wall paints. Beginning in 2027, Södra will become a kraft lignin producer and Stora Enso, which provides renewable products for packaging, biomaterials, and wooden construction, will be one of their first customer (https://www.Sodra.com/en/global/).

This thesis centres on the use of steam pretreated spruce for the release of sugars (C5 and C6) via enzymatic hydrolysis after which these sugars can be fermented to a wide variety of end products depending on the microorganism used.

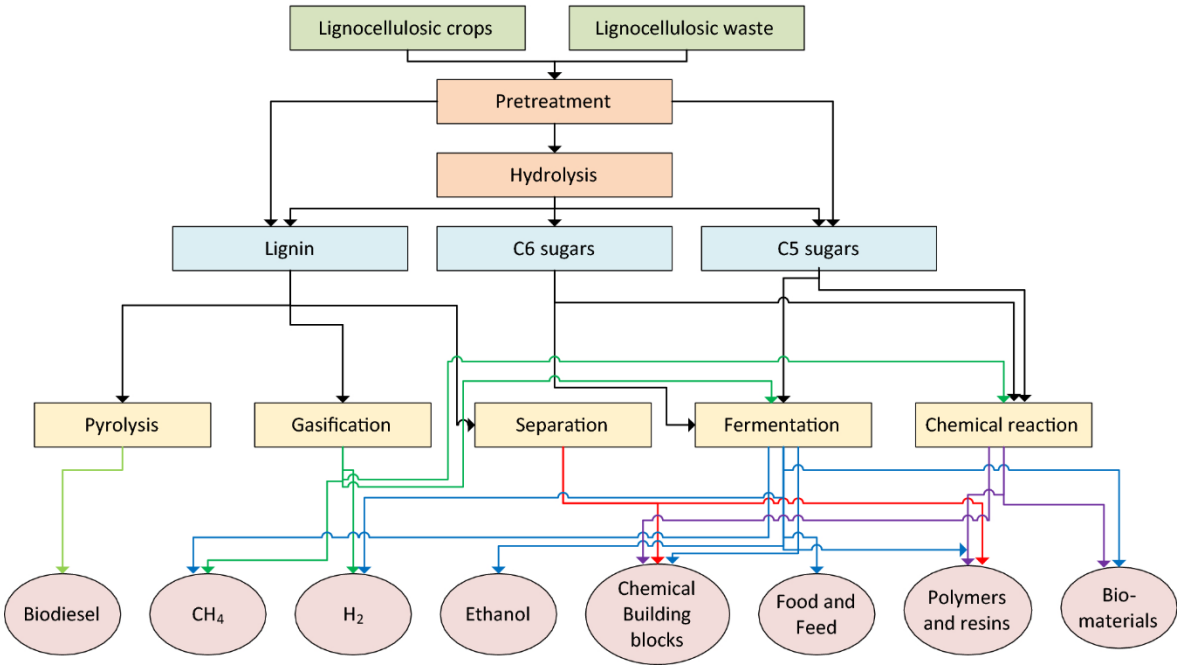

Figure 1.1. Schematic representation of a biorefinery producing energy carriers and chemicals. The figure was retrieved from Galbe & Wallberg, 2019 under CC BY 4.0 license.

1.2 Challenges in woody biomass saccharification

Woody biomass can be divided in two main groups: hardwood (i.e. birch) and softwood (i.e. spruce). Differences between the two will be presented in Chapter 2. In general, all woody biomass requires pretreatment prior to effective enzymatic saccharification. The pretreatment step is necessary to make the different carbohydrate polymers more accessible to hydrolytic enzymes (Chandra et al., 2007; Galbe & Zacchi, 2012). Moreover, to maximise the yield of valuable compounds found in lignocellulosic materials, pretreatment methods should enable efficient recovery of carbohydrates as well as lignin (Galbe & Wallberg, 2019).

Pretreatment methods encompass four main categories: physical, chemical, physico-chemical, and biological. Steam pretreatment (or steam explosion - STEX) is one of the most widely investigated methods and has the advantage of being easily applied to a wide range of lignocellulosic materials by simply adjusting pretreatment conditions (i.e. severity) (Chandra et al., 2007; Galbe &

Wallberg, 2019; Pielhop et al., 2016; Sun, et al., 2016). During steam pretreatment, hemicellulose is solubilized while lignin depolymerizes and recondenses in the solid fraction (Li et al., 2007). The extent of these outcomes depends on the conditions applied. In the case of spruce, achieving industrially relevant enzymatic saccharification requires harsh steam pretreatment conditions, which necessitate the addition of an acid catalyst (e.g. sulphur dioxide or sulphuric acid) (Wang et al., 2018). Following harsh pretreatment conditions, some of the solubilized hemicellulose is converted into compounds that inhibit either the hydrolytic enzymes or the microorganisms employed downstream in the process, and the exposed cellulose could be degraded (Martín et al., 2018; Sjulander & Kikas, 2020). Both events would contribute to yield losses of the process. Applying mild conditions during steam pretreatment can lower the chemical load of the process, resulting in a pretreated material with reduced hemicellulose solubilization and, consequently, less inhibitory compounds are formed. Also, mild steam pretreatment decreases the degree of lignin condensation enhancing its further processability (Pielhop, 2023). Studies have focused on optimising pretreatment parameters under mild conditions to achieve efficient enzymatic hydrolysis with low capital and operational costs (Jedvert, 2014; Moya et al., 2024; Yuan et al., 2021).

To maximise the saccharification step, different companies are developing enzymatic cocktails for the hydrolysis of lignocellulosic biomass. Novozymes (since 2024, renamed as Novonesis), based in Denmark, has released various products for lignocellulose saccharification including Cellic® CTec2, a blend of cellulases containing also high levels of β-glucosidases and hemicellulases activities (Novozymes application sheet, 2010). Cellic® CTec2 has become a benchmarck commercial cocktail for enzymatic hydrolysis in academia. **Table 1.1** provides an overview of glucose release from different biomass treated using Cellic® CTec2. Given that glucose release depends largely on the material and pretreatment used, tailor-made cocktails designed specifically for a certain substrate could maximise saccharification efficiency (Agrawal et al., 2018; Hu et al., 2015; Moya et al., 2024). In nature, filamentous fungi grow on and degrade lignocellulosic biomass. Characterising the lytic enzymes secreted by filamentous fungi could drive the design of such tailor-made cocktails (Filiatrault-Chastel et al., 2021; Varriale & Ulber, 2023). Clariant AG, a specialty chemicals company based in Switzerland, produces in-house the enzymes for saccharification depending on the lignocellulosic material that needs to be implied in the process (https://www.Clariant.com/en).

Table 1.1 Examples from the literature of glucose release after saccharification of differently pretreated biomass using Cellic® CTec2.

Biomass	Pretreatment	Time of hydrolysis	Enzyme loading	Glucose release	Reference
Wheat straw	Hydrothermal 195°C, 6/12 min	96 h	10 FPU/g cellulose	99% (w/w)	(Rodrigues et al., 2015)
Spruce	Sulphite pulping	48 h	4 mg_{enzyme}/g DM	72% (w/w)	(Müller et al., 2018)
Spruce	Steam pretreatment 210°C, 5 min (2.5% SO_2)	72 h	15 mg_{enzyme}/g cellulose	70% (w/w)	(Weiss et al., 2019)
Wheat straw*	Steam pretreatment 195°C, 18 min	72 h	15 mg_{enzyme}/g cellulose	65% (w/w)	(Weiss et al., 2019)
Poplar	Steam pretreatment 200°C, 5 min (3% SO_2)	48 h	14.2 FPU/g cellulose	90% (w/w)	(Sun et al., 2015)
Sweet sorghum bagasse	Steam pretreatment 180°C, 5 min (3% SO_2)	48 h	14.2 FPU/g cellulose	75% (w/w)	(Sun et al., 2015)
Wheat straw	Organosolv pretreatment 160°C, 40 min (diethylene glycol)	72 h	9 mg_{enzyme}/g DM	77% (w/w)	(Salapa et al., 2017)

*continuous reactor; FPU, filter paper unit; DM, dry mass

1.3 Aim and research questions

The aim of the present thesis was to build the foundation for tailor-made enzyme cocktails for saccharification of mildly steam pretreated spruce based on structural knowledge of the material before and after enzymatic hydrolysis. To this end, spruce was steam pretreated at two different temperatures (180°C and 210°C) with and without acid catalysts (acetic acid-HAc, H_2SO_4, H_3PO_4, and SO_2) to cover a wide severity range. The following research questions were formulated:

1. *What is the impact of decreasing pretreatment severity on the composition, cellulose ultrastructure, lignin condensation, and overall structure of spruce?*

Steam pretreatment was patented in 1926 (Mason, 1926) and, since then, the most efficient way to produce bioethanol from spruce has been to apply harsh pretreatment conditions, because the main focus was to produce ethanol from the cellulose fraction (Galbe & Zacchi, 2002; Pielhop et al., 2016). Nowadays, the need for material- and energy-efficient processes is driving biorefineries to maximise the use of all lignocellulosic constituents, including hemicellulose and lignin (Galbe & Wallberg, 2019). To reach such a goal, milder steam treatment is an alternative (Jedvert, 2012). At present, knowledge regarding the impact of lowering the severity of steam pretreatment on spruce is scarce (Bura et al., 2003). This knowledge gap is addressed in **Paper 1** and partially in **Paper 2**, where the structural characterisation of differently steam pretreated spruce is presented.

2. *How can structural and enzyme knowledge be combined to optimise cellulolytic cocktails with targeted enzyme supplementation?*

Enzymatic saccharification of lignocellulosic biomass is often one of the most expensive steps in biorefineries. Several substrate- or enzyme-related factors affect this process (Chandra et al., 2007), including hydrolysis conditions (i.e. pH or type of buffer), enzyme-substrate interaction, material composition, and cellulose crystallinity (Hu et al., 2014; Leu & Zhu, 2013; Mansfield et al., 1999). Consequently, understanding these factors, as well as enzyme functioning and biomass structure can contribute to the development of a highly specific tailor-made cocktail.

To answer research question 2, this thesis started out by gaining structural knowledge of steam pretreated materials (**Papers 1 and 2**) together with the definition of the base case hydrolysis with a cellulolytic cocktail (Celluclast® + Novozym® 188) (**Paper 1**). Combining the acquired data with known enzymatic properties (i.e. substrate specificity or optimal reaction conditions) pointed to single enzymes and a commercial cocktail to supplement the cellulolytic cocktail. Enzyme supplementation was evaluated based on sugar release and structural characterisation of the residual material after saccharification. This information was instrumental in the selection of other enzymes for cellulolytic cocktail supplementation (**Papers 2, 3, and 5**) as outlined in **Figure 1.2**.

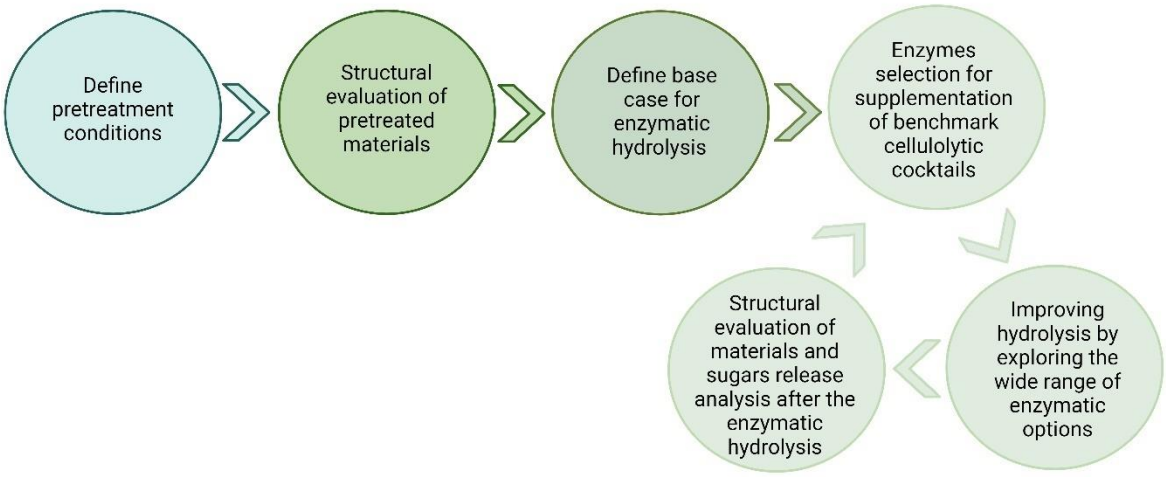

Figure 1.2. Flow chart of the approach used for enzyme supplementation in this thesis. The figure was made using BioRender.com.

3. *Is the secretome of filamentous fungi affected by the structure of the biomass on which they grow? How can such knowledge help optimise cellulolytic cocktails?*

Filamentous fungi grow on lignocellulosic biomass, which has led to the discovery of new enzymes or to the assembly of secretomes containing all the enzymes necessary to degrade a certain substrate (Filiatrault-Chastel et al., 2021). Filamentous fungi adjust their secretome through several mechanisms, which are affected by substrate structure and composition (Novy et al., 2019; Peciulyte et al., 2014; Tõlgo et al., 2021). A varied biomass composition implies the presence of different inducers, which could result in secretomes with a diverse content (i.e. composition or abundance). The present thesis sought to determine whether the severity of pretreatment impacted secretome composition. To this end, a filamentous fungus (*Thermothielavioides terrestris*) was grown on differently steam pretreated spruce, with similar composition but different structure, and its secretome content was assessed (**Paper 4**). The secreted enzymes were tested as supplement to the cellulolytic cocktail.

Chapter 2. Lignocellulosic feedstock

When conceptualizing a biorefinery, the availability of (cheap) raw materials should be taken in consideration together with many other aspects (de Jong, E. et al, 2011). Depending on the country, easily accessible lignocellulosic feedstock may include forest, agricultural or herbaceous resources and residues. In Sweden, forests cover 70% of the country, and harbour around 87 billion trees. There is now twice as much timber in Sweden as there was 90 years ago. About 1% of Sweden's forest is felled annually, and at least two new trees are planted for each tree that has been harvested. In terms of composition, softwood represent the vast majority, with Scots pine and Norway spruce accounting for 39% and 42%, respectively, of the total forest volume, while the remaining 19% is made of broad-leaf species (Swedish Forest Agency, 2018). Given its abundance, softwood (Norway spruce) was used as raw material in this thesis. In this chapter, the structural organisation and composition of softwood will be discussed with some parallelism between softwood and hardwood.

2.1. Softwood organisation

The softwood stem comprises different layers (**Figure 2.1**). The outer layer is called bark, and it protects the wood from physical, chemical, and biological degradation. The phloem consists of inner cells, and serves for the transport of nutrients, as well as storage. Further inside, the vascular cambium is a thin layer of cells, which generates phloem cells on its outside and xylem cells on its inside. The xylem constitutes the bulk of the wood material and is divided into sapwood (towards the outer part) and heartwood (towards the inner part). Sapwood is composed of living and dead cells; whereas heartwood is made entirely of dead cells. The centre of the stem is occupied by the pith, which contains tissues produced in the initial stages of plant growth. The xylem is normally organised into distinct concentrically orientated growth rings. Each of these rings is composed of earlywood and latewood cells, whose distinction is clearer in softwood than in hardwood. Earlywood cells have a larger cross-section and thinner walls than latewood cells. The cells in softwood are arranged in tracheids (longitudinal direction) and ray parenchyma (radial direction) (Geoffrey, 2009; Muzamal, 2016). In softwood, the tracheids are typically aligned in chains, with each line of cells derived from the same mother cell. In hardwood, the alignment is more complex because different cell types (e.g. vessels, fibres, and parenchyma cells) of varying sizes are produced. For instance, in softwood, fluids are conducted via earlywood tracheids; whereas in hardwood, this function is provided by specialized cells called vessels. Pits are one of the most characteristic microstructures in the cell wall of both softwood and hardwood. They represent canals that allow the flow of liquids

both laterally and vertically through the cell walls. The pits between tracheids are called bordered pits; those between tracheids and ray cells are called cross-field pits (Wiedenhoeft, 2010).

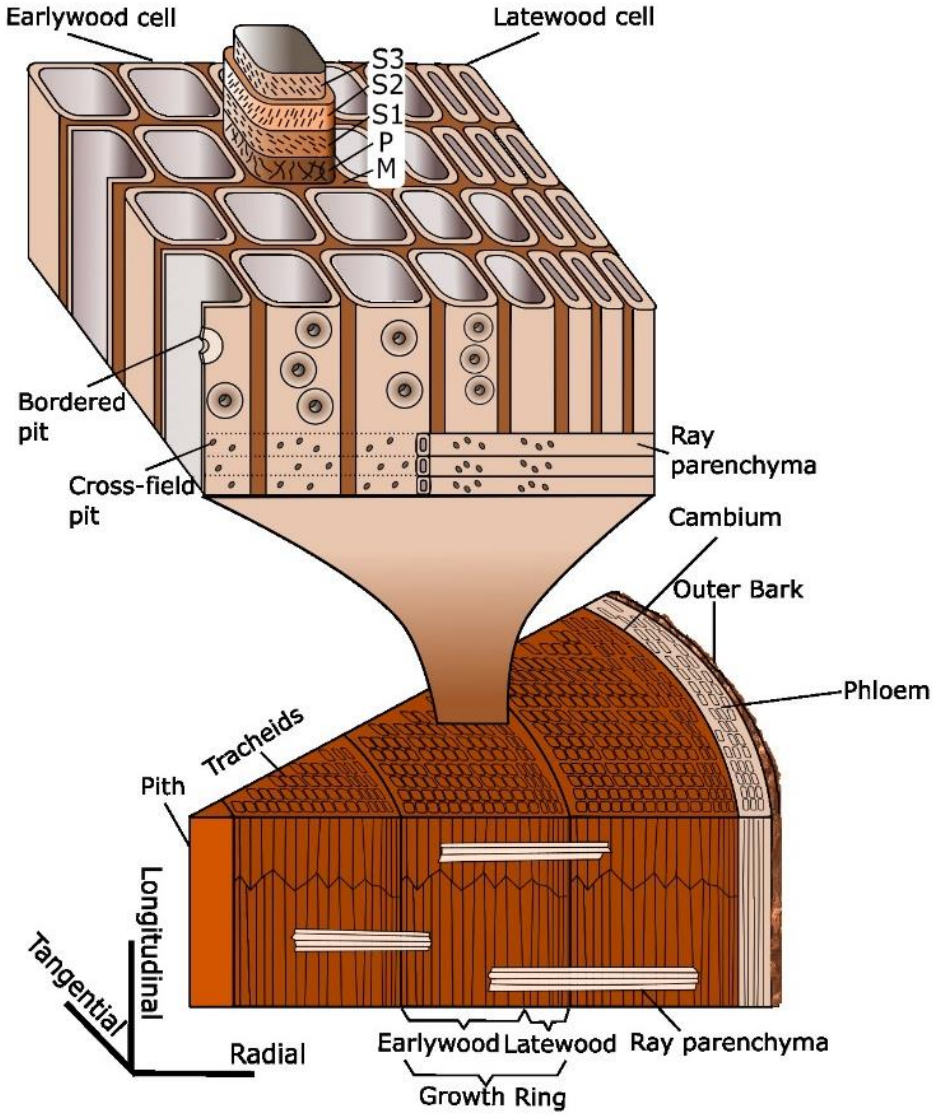

Figure 2.1. Organisation of the softwood cell wall. The figure was adapted from Muzamal, 2016 under CC BY 4.0 license**.**

2.2. Softwood cell wall constituents

The cell wall consists primarily of cellulose, hemicellulose, and lignin; however, it contains also small fractions of proteins, extractives, and inorganic compounds. The cells are joined together by the middle lamella (**Figure 2.1, M**) which is composed mainly of lignin. A closer look at a single cell reveals that the cell wall comprises a primary layer (**Figure 2.1, P**) and three secondary layers (**Figure 2.1, S1-S2-S3**) with different composition. The primary cell wall is dominated by hemicellulose and lignin, along with some cellulose (Srndovic, 2008). The secondary cell wall layers are characterised by different thickness and composition (Bodig, 1982; Brändström, 2001). Layers S1 and S3 (0.1–0.2 μm) are thinner than layer S2 (1–5 μm). S1 contains more lignin than S2 and S3; whereas S2 harbours more hemicellulose and cellulose compared to S1 or S3. Another difference is related to the orientation of cellulose microfibrils (see Section 2.3.1). These are oriented randomly in the primary layer and uniformly in the secondary layers. The cellulose microfibrils in S1 and S3 are almost perpendicular to those in S2 (Fernandes et al., 2011). The material composition presented in Chapter 3 refers to the average composition without differentiating between the layers in the plant cell wall.

2.2.1. Cellulose

Cellulose consists of anhydroglucopyranose monomers joined together by β-1,4-glycosidic bonds that form a linear polymer. Because each monomer is positioned at 180° with respect to the others, the basic repeating unit is a β-1,4-linked disaccharide of glucopyranose known as cellobiose (Monica Ek et al., 2009; Wohlert et al., 2022). Cellulose is polar, which means it has both a non-reducing and a reducing end. In the former, the terminal sugar is incorporated in a ring; whereas in the latter, it presents a free aldehyde or ketone group that can switch from a ring to a chain form (Albersheim et al., 2010).

Cellulose is organised into progressively more complex structures (**Figure 2.2**). It is common understanding that between 12 and 32 glucan chains (Fernandes et al., 2011) are held together by hydrogen bonds or van der Waals bonds to form elementary fibrils, or microfibrils (Kubicki et al., 2018). These microfibrils range from a high degree of order (i.e. crystalline regions) to a low degree of order (i.e. para-crystalline regions). The microfibrils can be entangled into larger macrofibrils covered by hemicellulose and lignin, which confer structure and protection to cellulose (Novy et al., 2019; Park et al., 2010; Wohlert et al., 2022).

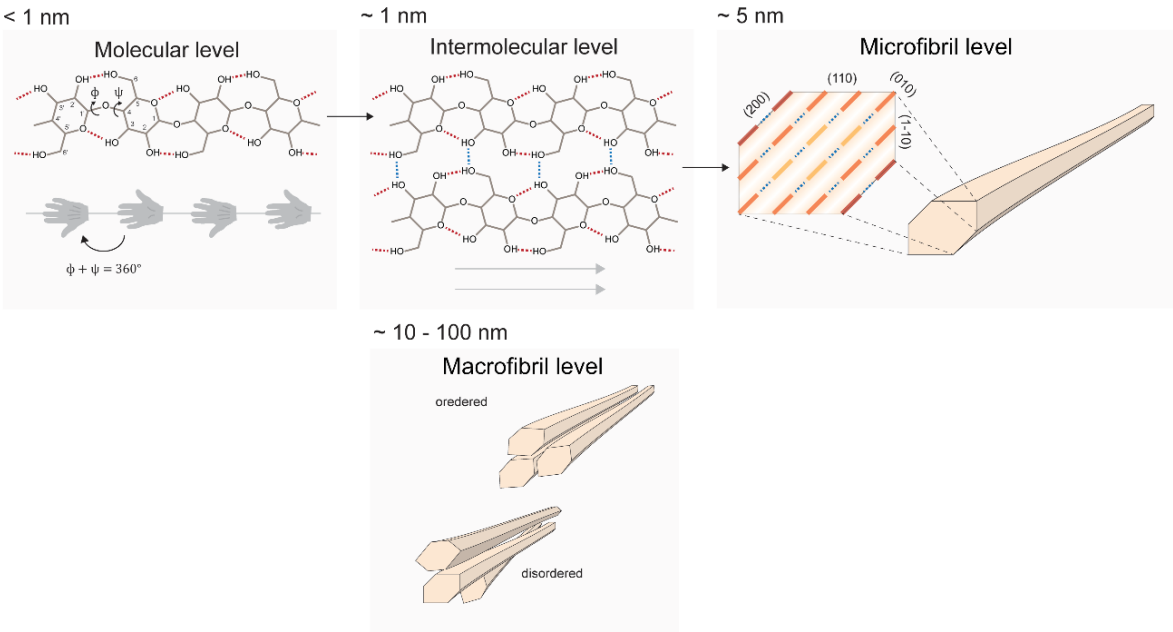

Figure 2.2. Schematic representation of the ultrastructural organisation of cellulose, with estimated dimensions. The figure illustrates the hierarchical organization of cellulose, from the molecular level to the arrangement of microfibrils. For simplicity hemicellulose and lignin are not represented at the microfibril level. The figure was adapted from Wohlert et al., 2022 under CC BY 4.0 license.

2.2.2 Hemicellulose

Hemicelluloses are heterogeneous polysaccharides characterised by a highly branched structure, which prevents an ordered organisation as in cellulose (Scheller & Ulvskov, 2010). Softwood cell wall hemicelluloses are composed of galactoglucomannan and arabinoglucuronoxylan, with the former being the most abundant (15–25% w/w) (**Figure 2.3**) (Terrett & Dupree, 2019). The spruce used for steam pretreatment (**Paper 1** and rest of this thesis) had an overall hemicellulose content of 20% w/w dry mass (DM).

The galactoglucomannan backbone is composed of β-1,4-linked glycosyl and mannosyl units. Carbons 2 and 3 of mannosyl residues can be acetylated; whereas carbon 6 can be galactosylated via α-1,6-linkages. There are two main types of galactoglucomannan in softwood: one that is high in galactose, with a galactose:glucose:mannose ratio of 1:1:3, and one that is low in galactose, with ratios of 0.1–0.2:1:3–4. The latter is sometimes referred to as glucomannan and is less soluble, given its lower degree of substitution (Laine, 2005; Lundqvist et al., 2003; Timell, 1967) (**Figure 2.3**).

Softwood xylan consists of a main chain of β-1,4-D-xylan, substituted by 4-*O*-methylglucuronic acid and arabinose units. These two are bound to the xylan backbone via α-1,2-linkages and α-1,3-linkages, respectively (**Figure 2.3**). Unlike softwood, hardwood hemicellulose is composed mainly of glucuronoxylan plus a small percentage (around 5% w/w) of galactoglucomannan and both polymers are heavily acetylated (Scheller & Ulvskov, 2010; Terrett & Dupree, 2019).

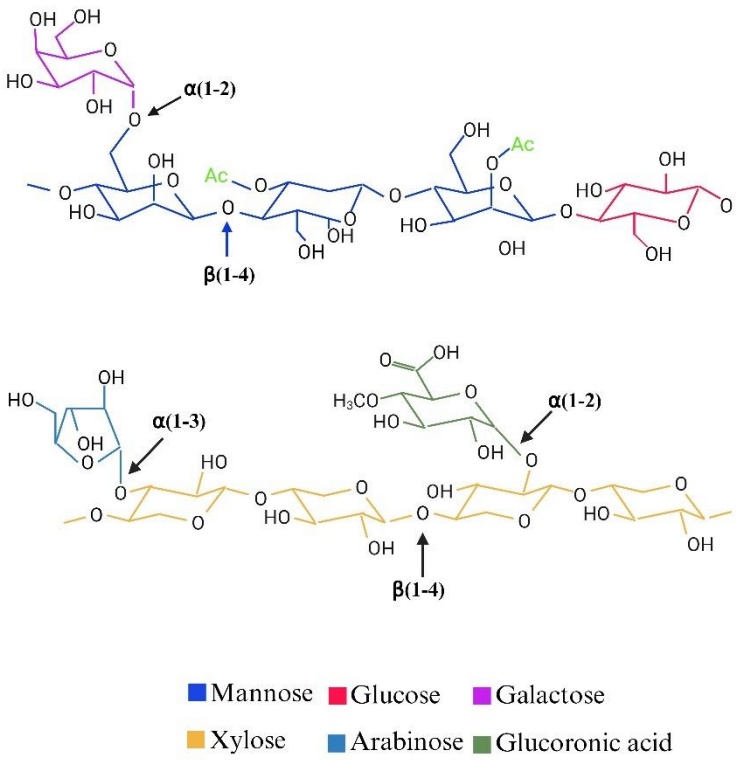

Figure 2.3. Chemical representation of arabinoglucurono-xylan (top) and galactoglucomannan (bottom). The figure was made using BioRender.com.

2.2.3 Lignin

The main function of lignin is to add strength and rigidity to the cell wall. Lignin is a highly heterogeneous biopolymer formed by the oxidative polymerization of multiple phenylpropane monomers. Syringyl (S), guaiacyl (G), and p-hydroxyphenyl (H) are the different monomers; they have two, one or no methoxy groups in the benzene ring, respectively. Monolignols are polymerized via phenoxy radicals formed by laccases and peroxidases, which oxidize the phenolic OH-groups.

Typically, softwood lignin is composed by G unit. Conversely, hardwood lignin contains mainly G and S units (Duval & Lawoko, 2014; Gellerstedt & Henriksson, 2008). The S/G ratio determines the hydrolyzability of woody biomass, but the exact reason for this still needs to be elucidated (see Section 5.2.2). One possibility seems to be related to the high affinity of G units for enzymes, which are then unproductively bound to lignin (Yuan et al., 2021). The main bonds in lignin are β-*O*-4 ether linkages, and they appear more often in softwood than hardwood. Carbon-carbon (β-β and β-5) bonds have also been detected in both softwood and hardwood, with higher proportions in the latter (Zhang et al., 2022).

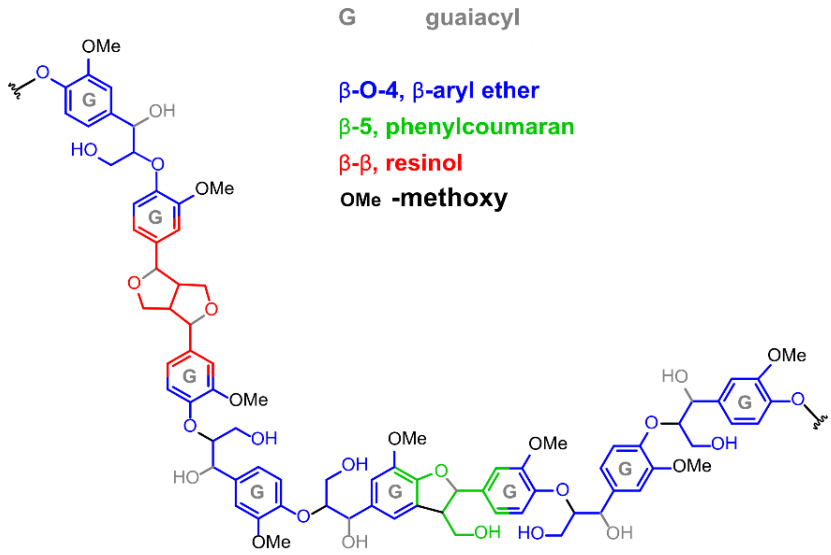

Figure 2.4. Schematic representation of softwood lignin showing β-*O*-4, β-β and β-5 linkages. The figure was adapted from Stewart et al., 2009 under CC BY 4.0 license.

Chapter 3. Biomass pretreatment

The purpose of biomass pretreatment is to change the chemical composition and structural organisation of the biomass, making it more susceptible to subsequent processing. The efficacy of pretreatment is increased by employing methods that maximise the yields of valuable compounds present in lignocellulosic materials (i.e. biomass fractionation). Pretreatment methods are typically divided into: physical (e.g. milling or grinding), chemical (e.g. alkali, acid, organic solvents, ionic liquids, and deep eutectic solvents), physico-chemical (e.g. steam pretreatments with or without catalyst, wet-oxidation, and hydrothermolysis), and biological (e.g. filamentous fungi) (Chandra et al., 2007; Chiaramonti et al., 2012; Galbe & Wallberg, 2019). **Table 3.1** lists the most common pretreatment methods used in the literature. Depending on the specific mechanism, the pretreated biomass may be differently suited for the next processing steps. In this thesis, steam pretreatment was selected to alter the structural and chemical makeup of the feedstock and render the cellulose component more amenable to enzymatic conversion. This chapter describes the features of steam pretreatment and the structural changes it induces on Norway spruce.

Table 3.1. Examples of pretreatment methods and their effect on biomass.
The table is adapted from Galbe & Wallberg, 2019**.**

Method	Active agent	Main effect on biomass
Dilute-acid pretreatment	H_2SO_4, H_3PO_4, and other strong acids	Hydrolysis of hemicelluloses
Alkali pretreatment	NaOH, lime, and similar alkaline compounds	Lignin solubilization
Steam pretreatment/steam explosion	High-temperature steam, with/without catalyst	Hydrolysis of hemicelluloses, particle size reduction
Ionic liquids	Large organic cation and a small inorganic anion	Fractionation of polymers
Hydrothermolysis	Liquid water under pressure at high temperatures, without catalyst	Hydrolysis of hemicelluloses
Organosolv	Organic solvents (e.g. ethanol or butanol), with/without catalyst	Lignin solubilization
Milling/grinding	Particle size reduction	Larger effective surface area
Biological treatment	Degradation of material	Depending on the fungus, they can degrade cellulose, hemicellulose, and/or lignin

3.1. Overview of steam pretreatment

Steam pretreatment (or STEX) is widely applied to lignocellulosic biomass. The technology originates from the paper and pulp industry, and was patented in 1926 by W.H. Mason (Mason, 1926). Since then, more than 100,000 scientific papers have been published on this method (number of entries on google scholar by searching for steam pretreatment or steam explosion from 1926 to 03/11/2024). By now, it offers a mature and flexible technology because a range of different lignocellulosic feedstocks can be pretreated successfully by adjusting the operating conditions (Mosier et al., 2005; Pielhop et al., 2016; Wang et al., 2018). The energy requirements are low compared to many other pretreatments. Holtzapple et al. showed that conventional milling techniques applied to aspen wood, required much higher energy input (70%) than steam explosion to reach a comparable size reduction of the feedstock (Holtzapple et al., 1989).

Steam pretreatment can be performed in either a batch or continuous reactor (Zimbardi et al., 1999). In this thesis, a batch reactor was used. The equipment used for steam pretreatment is known as a “steam gun” (**Figure 3.1**). Pretreatment starts by impregnation of the feedstock, which ensures the desired humidity content and catalyst concentration, if any. Then, biomass is treated with steam for a specified period (i.e. residence time) in the reactor. High-pressure steam is injected at the bottom and top of the reactor to ensure homogeneous conditions. Temperature and pressure increase inside the reactor and biomass is heated up by condensing heat. This causes the release of acidic substituents (e.g. acetyl groups) from hemicellulose, which lowers the pH in the reactor and causes hemicellulose solubilization (autocatalysis) (Mosier et al., 2005; Pielhop et al., 2016). Because different lignocellulosic feedstocks contain different amounts of acetyl groups in hemicellulose (Terrett & Dupree, 2019), acidity during pretreatment affects the extent of hemicellulose solubilization. This stage influences also the structure of lignin, with the degradation of β-*O*-4 ether bonds (for details, see Section 3.2) (Gellerstedt & Henriksson, 2008; Pielhop et al., 2015). When the residence time is over, the pressure in the reactor is released abruptly by opening a reactor valve and the biomass is discharged into a blow tank. Because of the sudden drop of pressure, down to atmospheric pressure, flashing of superheated water occurs, which causes defibration of the steam treated wood chips and disrupts the cell wall architecture. This structural collapse is further amplified by the impact of biomass against the vessel walls when it is discharged in the blow tank (Pielhop et al., 2016).

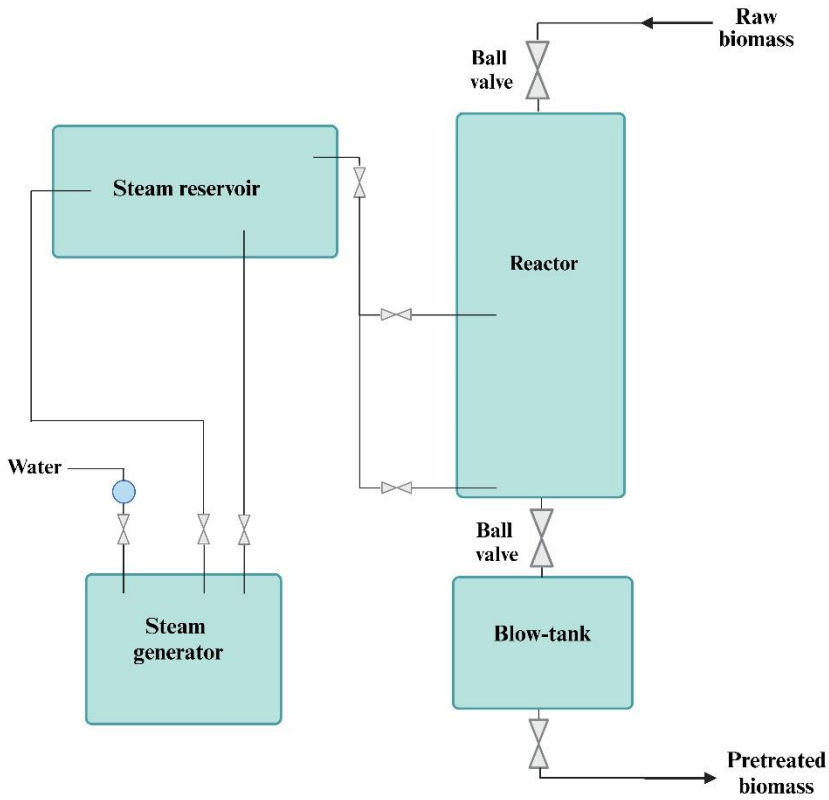

Figure 3.1. Schematic representation of the steam gun. The figure was made using BioRender.com.

Pretreatment conditions, such as temperature, residence time, and catalyst, vary according to the selected lignocellulosic biomass and the subsequent application for the pretreated material. To rank different pretreatment conditions, the aggregate pretreatment severity factor R_0 can be used (Chum et al., 1990; Kim et al., 2014). R_0 is calculated by equation 1, where T_r is the reaction temperature (°C) and t is the reaction time (min).

$$\boldsymbol{R_0 = t * \exp\left(\frac{T_r - 100}{14,75}\right)} \qquad (1)$$

However, steam pretreatments performed under the same temperature and residence time, but different catalysts or catalyst concentrations, cannot be differentiated by R_0. In these instances, it is better to use the aggregated combined severity factor (CSF) metric (Chum et al., 1990), which is based on R_0 and incorporates the pH value of the slurry after pretreatment (equation 2).

$$\boldsymbol{CSF = logR_0 - pH} \qquad (2)$$

Usually, steam pretreatment conditions for Norway spruce are 5–10 min residence time, 200–220°C, and up to 4% (w/w) acid catalyst (Galbe & Wallberg, 2019; Galbe & Zacchi, 2012; Wang et al., 2018). The low content of acetyl groups in softwood hemicelluloses necessitates an acid catalyst during steam pretreatment. The most common acid catalysts for steam pretreatment of Norway spruce are H_2SO_4 and gaseous SO_2 (Galbe & Zacchi, 2002; Wang et al., 2018; Zhong et al., 2023). Although these conditions allow significant glucose release after enzymatic hydrolysis, strong acids and elevated temperatures cause the partial conversion of cellulose into oligo- and monosaccharides. These compounds can then be degraded further to their dehydration products, i.e. furfural, 5-hydroxymethylfurfural, levulinic acid, and formic acid, which inhibit microbial growth during the fermentation step and cause a loss of fermentable sugars (Martín et al., 2018; Sjulander & Kikas, 2020). Moreover, high chemical loads are associated with additional costs for inputs and neutralization, as well as their recycling, if possible.

To overcome the drawbacks posed by harsh conditions, milder pretreatments can be attempted by decreasing the chemical load of the process and, therefore, decreasing the formation of inhibitory compounds (Jedvert et al., 2012). Such rationale represented the basis for the selection of pretreatment conditions in this thesis. Briefly, spruce was steam pretreated at 180°C and 210°C, with or without the addition of various acidic catalysts (HAc, H_3PO_4, H_2SO_4, and SO_2). The CSF was used in **Paper 1** to rank and compare the different conditions used for steam pretreatment. The CSF ranged from 0.66 for mild $STEX_{180°C/auto}$ to 3.1 for severe $STEX_{210°C/SO2}$ pretreatment, which coincided with a drop in pH from 3.9 to 1.5 in the resulting slurry (**Table 3.2**). Except for $STEX_{210°C/SO2}$, in all other pretreatment conditions, the materials retain different amounts of hemicellulose (for details, see Section 3.2).

Table 3.2 Summary of the conditions applied for pretreatment of Norway spruce biomass and combined severity factor (**paper 1)**.

	$STEX_{180°C/auto}$	**$STEX_{210°C/auto}$**	**$STEX_{210°C/HAc}$**	**$STEX_{210°C/H3PO4}$**	**$STEX_{210°C/H2SO4}$**	**$STEX_{210°C/SO2}$**
Temperature (°C)	180	210	210	210	210	210
Residence time (min)	5	5	5	5	5	5
Catalyst[a]	autocatalyzed	autocatalyzed	1% HAc	0.1% H_3PO_4	0.1% H_2SO_4	3% SO_2
pH liquid fraction	3.9	3.6	3.1	2.9	2.5	1.5
CSF	0.7	1.0	1.5	1.7	2.1	3.1

[a]The percentages are expressed in w/w.

HAc, acetic acid.

3.2. Structural changes in steam pretreated spruce

Structural changes in steam pretreated spruce result from the interplay between different chemical and physical modifications taking place during pretreatment. When the biomass is in the reactor at high temperature, pressure, and acidic pH, the glycosidic bonds in hemicellulose are hydrolysed. The extent of these reactions increases with the severity of pretreatment, ultimately leading to cellulose hydrolysis at elevated severity (Chandra et al., 2007; Muzamal, 2016). Cellulose is harder to degrade than hemicellulose due to the presence of hydrogen bonds and van der Waals bonds between the glucan chains in the cellulose microfibril (Wohlert et al., 2022). During this stage of the pretreatment, also the lignin structure is altered, undergoing through cycles of depolymerization and recondensation (Li et al., 2007). The β-*O*-4 ether bonds in lignin are degraded under acidic conditions and when the temperature of the pretreatment is higher than the lignin glass transition temperature (140–160°C) (Börcsök & Pásztory, 2021) it enables the lignin redistribution during the decompression. Thus, at the end of the residence time, the plant cell wall results altered compared to the raw material. Further modifications arise following explosive decompression and the impact of biomass against the vessel walls. Pielhop et al., reported that the explosion step led to material with a more porous surface and smaller particle size compared to steam treated spruce without explosion (Pielhop et al., 2016). Similar results were obtained by Muzamal who studied the effect of temperature, explosion, and impact of wood chips against the vessel walls during steam pretreatment (Muzamal, 2016). The extent of chemical and physical modifications depends on the severity of the pretreatment and have been studied in detail for harshly steam pretreated spruce. **Papers 1 and 2** describe the impact of less severe pretreatment on spruce, with the key findings presented in Sections 3.2.1 and 3.2.2.

3.2.1 How are hemicellulose, cellulose, and lignin in spruce affected by lowering steam pretreatment severity?

The present section discusses the impact of lowering the severity of steam pretreatment on the hemicellulose composition, cellulose ultrastructure, and lignin degradation in spruce. As reported in **Table 3.3** and in **Paper 1**, hemicellulose content decreased from 20% w/w DM in the raw material to 0% w/w DM in $STEX_{210°C/SO2}$. Solubilization of arabinose on arabinoglucuronoxylan was observed in all pretreatments. Almost complete loss of galactose on galactoglucomannan occurred already with $STEX_{210°C/auto}$. During $STEX_{210°C/HAc}$, $STEX_{210°C/H3PO4}$, and $STEX_{210°C/H2SO4}$, the content of xylan decreased from 5.7% to 0.7% w/w DM and that of glucomannan from 13.2% to 0.6% w/w DM with increasing severity (**Table 3.3A-B**). Thus, a more severe pretreatment caused less hemicellulose to be retained in the solid fraction and also decreased its complexity (in terms of substituents).

Hemicellulose solubilization affects cellulose ultrastructure (**Paper 1, Figure 3**), as evidenced by small-angle and wide-angle X-ray scattering (SAXS/WAXS) (for details, see Section 4.3). Increased severity of pretreatment led to a slight increase in crystallite dimension, as observed by WAXS, suggesting cellulose microfibrils aggregation. Concomitantly, SAXS revealed an increase in the distance between the microfibrils' centre. Considering that WAXS indicated microfibrillar aggregation, it is possible that the distance among microfibrils (SAXS) is between cellulose microfibril aggregates instead, as the microfibrils are closely packed inside the aggregates and give rise to similar scattering. Such change can derive from the removal of hemicellulose, which acted as a spacer between microfibrils.

The structural alterations in cellulose may relate also to the depolymerization and recondensation of lignin. Lignin gives strength and rigidity to the cell wall; therefore, its relocation would alter the cell wall structure (Takada et al., 2019). The effect of acid-catalyzed steam pretreatment on lignin chemical bonds was evaluated by two-dimensional heteronuclear single quantum coherence nuclear magnetic resonance (2D HSQC-NMR) (for details, see Section 4.2); whereas lignin recondensation was evaluated by scanning electron microscopy (SEM). Increasing the severity of pretreatment, a decrement of β-O-4 linkages was observed. A similar trend was observed for β-5 linkages, although to a lesser degree, demonstrating the stable nature of lignin carbon-carbon bonds, including β-β linkages, under acidic conditions. Notably, signals for covalent bonds between hemicellulose and lignin (so called lignin carbohydrate complexes or LCCs) were also detected but, except for the results presented in **Paper 1**, LCCs were not further investigated (**Paper 1, Table 3**) (Tarasov et al., 2018; Terrett & Dupree, 2019). In **Paper 2**, the relocation of lignin was evaluated using SEM. During steam pretreatment, lignin relocated to form droplets (Donohoe et al., 2008; Takada et al., 2019), whose appearance on the surface became more frequent with pretreatment severity. Lignin droplets were detected on the surface of both $STEX_{210°C/auto}$ (**Paper 2, Figure 4A**) and $STEX_{210°C/SO2}$ (**Paper 2, Figure 4B**) samples, although they were more numerous and larger in the latter. Therefore, increasing pretreatment severity leads to greater alterations in the structure of lignin and hemicellulose, significantly modifying the cell wall.

Table 3.3 A-B. Compositional analysis of lignocellulosic compounds in spruce before and after pretreatment. Steam pretreatment was performed with two different batches of raw material (A-B). Data represent mean values ± standard deviation of triplicates. The glucan amount corresponds to the amount of glucose from cellulose and from galactoglucomannan (**Paper 1**).

A	**Raw material (%w/w DM)**	**STEX$_{180°C/auto}$ (%w/w DM)**	**STEX$_{210°C/auto}$ (%w/w DM)**	**STEX$_{210°C/SO2}$ (%w/w DM)**
Glucan	45.6 ± 0.7	49 ± 1	52 ± 2	50 ± 2
Xylan	5.0 ± 0.1	4.9 ± 0.6	2.4 ± 0.1	0.0 ± 0.0
Arabinan	0.5 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Galactan	1.7 ± 0.1	1.5 ± 0.1	0.2 ± 0.0	0.0 ± 0.0
Mannan	12.1 ± 0.1	10.7 ± 0.6	4.1 ± 0.2	0.0 ± 0.0
ASL	2.6 ± 0.1	2.3 ± 0.1	1.4 ± 0.0	1.3 ± 0.0
AIL	32.4 ± 0.1	34.4 ± 0.6	41.4 ± 0.4	51.4 ± 0.5
Ash	BDL	BDL	BDL	BDL
Recovery	100 ± 1	103 ± 2	101 ± 1	103 ± 2

B	**Raw material (%w/w DM)**	**STEX$_{210°C/HAc}$ (%w/w DM)**	**STEX$_{210°C/H3PO4}$ (%w/w DM)**	**STEX$_{210°C/H2SO4}$ (%w/w DM)**
Glucan	45.5 ± 0.9	60.5 ± 0.3	61.3 ± 0.4	61.0 ± 0.1
Xylan	5.7 ± 0.1	1.7 ± 0.0	1.3 ± 0.0	0.7 ± 0.0
Arabinan	0.7 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Galactan	1.7 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Mannan	13.2 ± 0.1	1.2 ± 0.0	0.9 ± 0.0	0.6 ± 0.0
ASL	3.7 ± 0.1	2.9 ± 0.1	2.8 ± 0.1	2.7 ± 0.1
AIL	26.4 ± 0.2	36.0 ± 0.1	36.9 ± 0.1	38.2 ± 0.4
Ash	BDL	BDL	BDL	BDL
Water extractives	3.8 ± 1.5	-	-	-
Ethanol extractives	0.8 ± 0.1	-	-	-
Recovery	102 ± 3	103 ± 1	103 ± 1	103 ± 1

AIL, acid-insoluble lignin; ASL, acid-soluble lignin; BDL, below detection limit.

3.2.2 Physical modifications of steam pretreated spruce

Alterations in cellulose, hemicellulose, and lignin deconstruct the plant cell wall to an extent that depends on the severity of pretreatment. The expansion of vapour inside the tracheids when the pressure drops to atmospheric pressure, causes the formation of pores on the surface of wood chips (Pielhop et al., 2016). Then, when the biomass is discharged into a blow tank, cracks form between tracheids and defibration of steam treated wood chips is observed. The impact of wood chips against the vessel walls enlarges the cracks between tracheids and diminishes particle size, contributing to an overall decrease in particle dimensions of the materials (Muzamal, 2016; Pielhop et al., 2016). As exemplified in **Figure 3.2** for the differently steam pretreated materials used in this thesis, an overall decrease in particle size occurred with increasing the severity of the pretreatment.

Paper 2 examined the impact of lowering the severity of pretreatment on the surface of spruce biomass. A comparison of raw material surface with steam pretreated ones, revealed clear cracks between tracheids in all pretreated substrates, and an increase in surface defibrillation at increasing severity of pretreatment (**Paper 2, Figures 1A-B, 2, and 3**). The addition of a catalyst during pretreatment altered profoundly the structure of lignocellulosic biomass with a more defibrillated surface and larger cracks between tracheids (red arrows in **Figure 3.3C–F**). In the $STEX_{210°C/SO2}$ (**Figure 3.3F**), the overall structure almost collapsed, due to complete hemicellulose solubilization along with greater alteration of cellulose and lignin compared to the other catalyzed materials. Surface roughness augmented also dramatically with increasing severity of pretreatment (**Paper 2, Figure 3**) and cracks were observed in the pits of materials subjected to pretreatment with a catalyst (**Paper 2, Figure S1**), in line with earlier reports (Muzamal, 2016; Y. Zhang & Cai, 2006; Zhong et al., 2023).

Figure 3.2. Images of the different pretreated materials. (A) raw material, (B) $STEX_{180°C/auto}$, (C) $STEX_{210°C/auto}$, (D) $STEX_{210°C/HAc}$, (E) $STEX_{210°C/H3PO4}$, (F) $STEX_{210°C/H2SO4}$, and (G) $STEX_{210°C/SO2}$.

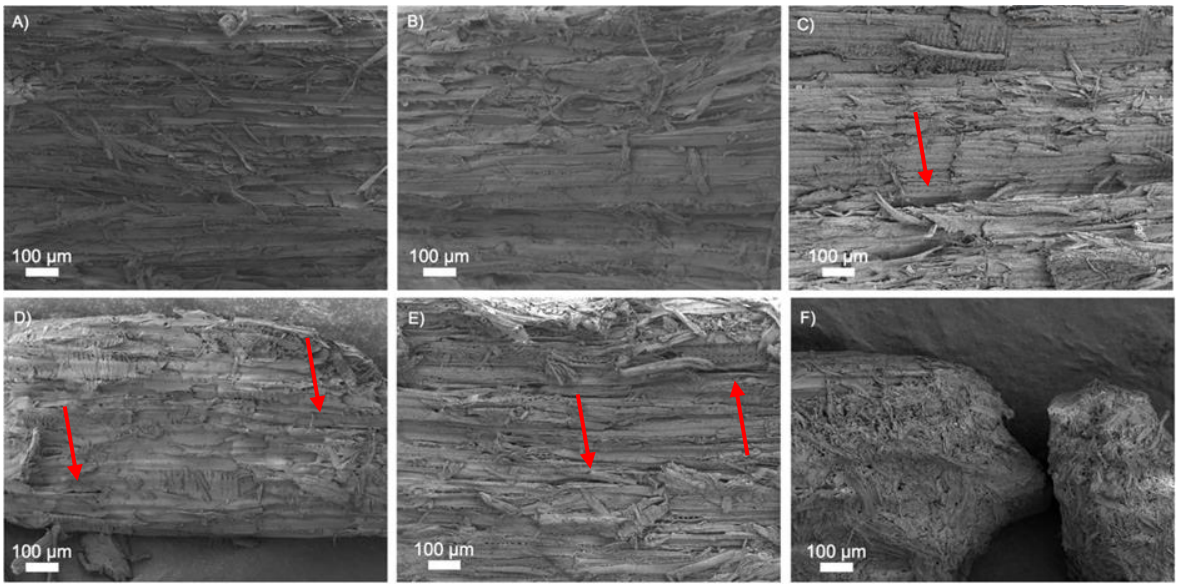

Figure 3.3. SEM images obtained at low magnification (100×) using the secondary electron signal from Norway spruce wood subjected to different pretreatments. (A) $STEX_{180°C/auto}$, (B) $STEX_{210°C/auto}$, (C) $STEX_{210°C/HAc}$, (D) $STEX_{210°C/H3PO4}$, (E) $STEX_{210°C/H2SO4}$, and (F) $STEX_{210°C/SO2}$. Red arrows point to cracks on the surface.

Key aspects of different steam pretreated materials:

- Hemicellulose complexity (in terms of substituents) and residual amount in the materials decrease increasing the severity of the pretreatment

- Cellulose microfibrils aggregation increases with severity and correlates with hemicellulose solubilization and lignin depolymerization and recondensation

- Alterations in lignin were more pronounced (decrement of β-O-4, β-5 linkages) with the addition of an acid catalyst, and lignin droplets were observed on the surface of $STEX_{210°C/auto}$ and $STEX_{210°C/SO2}$ with higher amounts in the latter

- Investigating the material's surface using SEM showed clear cracks between tracheids in all pretreated substrates. An increase in surface defibrillation and roughness was observed with increasing pretreatment severity.

Chapter 4. Analytical methods for biomass characterisation

This chapter reviews some of the analytical methods used during this thesis work for the structural characterisation of lignocellulosic biomass following pretreatment and enzymatic hydrolysis (**Table 4.1**). The resulting information elucidates how pretreatment conditions or additionally supplemented enzymes (**Chapter 5**) affect the individual biomass polymers.

Biomass was evaluated before and after enzymatic hydrolysis using SEM, SAXS, WAXS, and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). NMR was used for lignin analysis after pretreatment and Fourier-transform infrared spectroscopy (FTIR) for lignin surface analysis after enzymatic hydrolysis. By applying these different methods, the aim was to build a foundation for deeper understanding of the effect of lowering steam pretreatment severity and guide enzymatic hydrolysis set-up.

Table 4.1. Summary of the methods used for the structural evaluation of spruce.

Technique	Analytical methods	Structural information	Paper
Electron microscopy	SEM and FIB-SEM	Overall structural evaluation of biomass (tracheids arrangement and lignin droplets)	Papers 1 and 2
NMR	2D HSQC-NMR and heteronuclear multiple bond correlation	Analysis of lignin (intermonolignol bonds and lignin carbohydrate complexes)	Paper 1
X-rays scattering	SAXS and WAXS	Cellulose ultrastructure analysis (crystallinity index and distance between microfibrils)	Papers 1, 2, 3
Ion chromatography	HPAEC-PAD	Analysis of monosaccharides and oxidized oligosaccharides	Papers 1-5
FTIR	ATR-FTIR	Lignin surface analysis	Paper 5

4.1. Scanning electron microscopy

Electron microscopy generates high-resolution images of biological and non-biological samples. There are two main types of electron microscope – the transmission electron microscopy (TEM) and the scanning electron microscopy (SEM). In this work, SEM was used since TEM requires a more complex sample preparation (i.e. cutting the sample in thin slices) that would contribute to structural changes in the samples.

SEM is a technique which is usually chosen to characterise the material's surface. This technique uses an electron beam with acceleration voltages of up to 30 kV focused on the sample. Interactions between the electron beam and the sample emit signals collected by the detector, which combines them to form an image. SEM is a highly versatile tool for the structural characterisation of bio-based materials although these materials are composed of repeating units of low-atomic-number elements, such as carbon, hydrogen, nitrogen, and oxygen. Low-atomic-number elements interact less with accelerated electrons, resulting in in poor contrast and, therefore, the need for coating (usually made of gold) to study the sample (Herrero et al., 2023).

Within this thesis, SEM was used to evaluate the overall structure of differently steam pretreated spruce (**Paper 1, Figure 4**), as well as to identify detailed structural changes following pretreatment or enzymatic hydrolysis (**Paper 2**). To this end, the samples in **Paper 2** were coated with 5 nm Au.

SEM can also be coupled to a focused ion beam (FIB-SEM), which can then be used to remove ultrathin layers of tissue/material. In FIB-SEM, a dual-electron beam intersects the ion beam at 52° just above the sample's surface, producing instantaneous SEM imaging of the surface altered by the FIB. In **Paper 2**, FIB-SEM was used to analyse the material's cross section and evaluate the internal organisation of tracheids in the raw material (**Paper 2, Figure 1D**), $STEX_{210°C/auto}$, and $STEX_{210°C/SO2}$ (**Paper 2, Figure 4C-D**).

4.2. 2D-Nuclear magnetic resonance

NMR is the most common method for lignin analysis. It detects chemical structures by assessing local magnetic fields around atomic nuclei (nuclei with an odd atomic number). The spins of these nuclei are randomly oriented, but when placed in an external magnetic field, the spins will align parallel or antiparallel to the field. If the nuclei are subjected to electromagnetic radiation of the correct frequency, the nuclei, aligned with the external field, will absorb energy and "spin-flip"

to align themselves against the field. When the spin returns (relaxes), radio waves are emitted. However, as the magnetic properties of a nucleus depend on its chemical environment, the emitted radio wave frequency of a chemically bound nucleus will be shifted compared to that of a free nucleus. This so-called "chemical shift" is used to identify the chemical composition of a molecule (Ralph & Landucci, 2010; Sette et al., 2011).

In complex materials such as lignocellulose, conventional NMR does not allow detailed assignment and quantification of individual signals due to broad and overlapping NMR peaks, which result in low-resolution spectra (Vuković & Tišma, 2024). To overcome low-resolution spectra, it is possible to combine the sensitivity of two different NMR methods (2D-NMR) (Ralph & Landucci, 2010). In **Paper 1**, HSQC and heteronuclear multiple bond correlation (HMBC) 2D-NMR were applied for the analysis of lignin and LCCs (**Paper 1, Table 3**). HSQC correlates the chemical shifts of directly bound nuclei (normally ^{1}H and ^{13}C), where the chemical shift of the carbon lies in one dimension (F1) and the chemical shift of its attached proton in the other dimension (F2). However, the signal assigned to the γ-ester is still heavily overlapping with other lignocellulosic structures, and the detection of benzyl esters has seldom been reported (Balakshin et al., 2007; Capanema et al., 2014; Giummarella & Lawoko, 2017). When the signal of interest overlaps with other signals, HMBC comes into play as it allows to detect the bonds that are in close proximity to the target one. HMBC detects long-range correlations between heteronuclei that are not directly bonded but are separated by two or three bonds (Ralph & Landucci, 2010).

4.3. Small and wide angle X-rays scattering

X-ray scattering is a fingerprint technique for the analysis of molecular structures ranging from materials to proteins. The sample is irradiated with incident X-rays, upon which the scattering angle and intensity of the X-rays leaving the sample are measured. The diffraction pattern and intensity can be correlated directly with specific crystalline components in the sample, giving a picture of the crystal phase, as well as details on defects and deformities within the material that deviate from the perfect crystal structure. SAXS and WAXS differ with respect to the angle at which the scattered X-rays are measured: small angles ($2\theta = 0\text{-}5°$) for SAXS and wide angles ($2\theta > 5°$) for WAXS (Wu & Stacey, 2021). SAXS and WAXS can be used for the characterisation of cellulose ultrastructure after pretreatment (**Paper 1**) or enzymatic hydrolysis (**Papers 2 and 3**) (Penttilä et al., 2020). Cellulose ultrastructure can also be determined by solid state ^{13}C CP–MAS NMR, but the disadvantage of that technique is that the preparatory steps might alter the sample's architecture (Sun et al., 2014; Virtanen et al., 2015). SAXS and WAXS do not require any sample preparation (not even drying), but data analysis can be rather challenging due to X-ray scattering from water or hemicellulose. Nevertheless,

WAXS makes it possible to compute the crystallinity index from the total intensity and average size of crystallites in a material based on peak broadening in the scattered pattern (**Figure 4.1**). Instead, SAXS can be used to compute microfibril-to-microfibril distances.

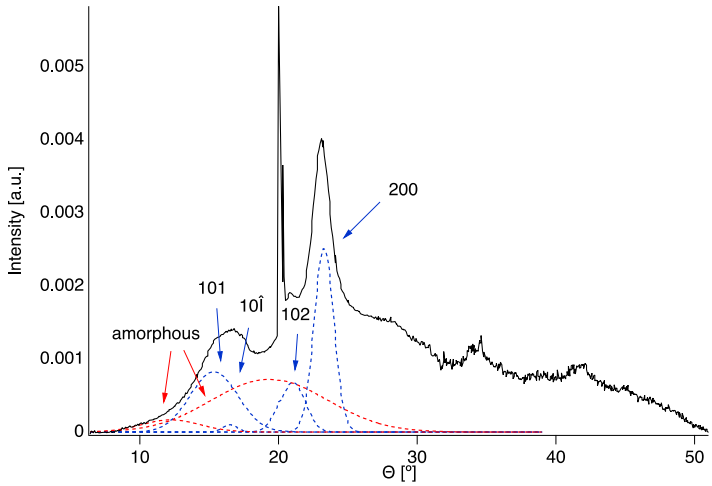

Figure 4.1. WAXS diffractogram of a spruce sample treated with H_2SO_4. Dashed lines represent theoretical fits to the experimental data. The WAXS profile consisted of five characteristic crystalline peaks (101, 10î, 102, 200, and 004) and a broad amorphous region at 19°. The curve above 26° was dominated by the scattering from water. A sharp peak at around 21° corresponds to the scattering from mica windows (**Paper 1**).

4.4. Ion chromatography

Ion chromatography separates ions and polar molecules based on their charge. The charged analytes of interest are separated on a column after which the eluted species are measured by a detector. Ion chromatography can be applied for the determination of ionic solutes, such as inorganic anions, cations, transition metals, and low-molecular weight organic acids and bases. HPAEC-PAD has been developed specifically for carbohydrate analysis. Carbohydrates are not charged, but they are weak acids with a pKa of 12–14. This means that at high pH (~12), neutral sugars become partially ionized and can be separated (Rohrer, 2013).

In this thesis, HPAEC was used to quantify sugar release (monosaccharides) and to detect oxidized products generated by lytic polysaccharide monooxygenases (LPMOs) (for details, see **Chapter 5**). Owing to the lack of commercial standards, the oxidized products (C4-ox, see Section 5.1.1) could only be analysed in a qualitative way. Therefore, as discussed in **Papers 3 and 5**, the oxidized products served to confirm that the LPMOs added during the hydrolysis was active. Peak height was compared to evaluate if the oxidized products were being accumulated or degraded during

hydrolysis (**Paper 3, Figure 4 and Paper 4, Figure 4**). As showed in **Figure 4.2**, the retention times of oxidized products changed over time, which could be ascribed to the sample matrix or heterogeneous eluents. When this happened, the oxidized standards were added to the samples to ensure that only one peak was present around the retention time of the standard, thereby confirming the presence of LPMO products (data not shown).

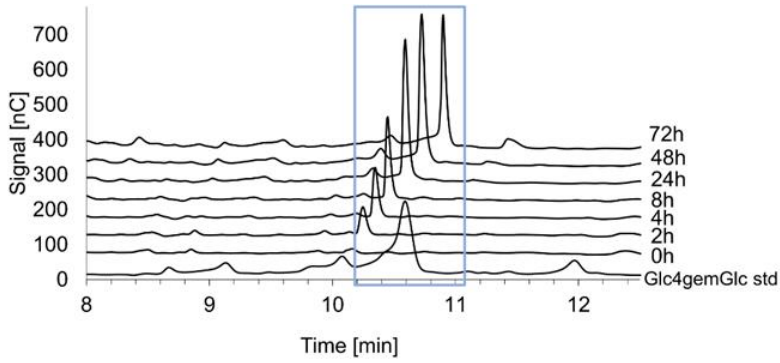

Figure 4.2. HPAEC-PAD chromatograms of LPMO oxidized products (Glc4gemGlc) over time. Analysis of the supernatant after enzymatic hydrolysis of $STEX_{210°C/SO2}$ with Celluclast® + Novozym® 188 supplemented with *Ta*LPMO9A.

4.5. Fourier-transform infrared spectroscopy

FTIR is a rapid and effective spectroscopic technique employed to identify and characterise functional groups in a material. The sample is irradiated with infrared radiation (4000–400 cm^{-1}), with a longer wavelength and a lower frequency than those of visible light. The radiation is absorbed when its frequency matches the vibrational frequency of a chemical bond. The absorbed energy causes a temporary change in the dipole moment of the molecule. This absorption can be detected and used to identify functional groups based on their characteristic vibrational frequencies (Herrero et al., 2023; Tiquia-Arashiro et al., 2023) A typical infrared spectrum is a plot of the absorbance (or transmittance) of the radiation through the molecule versus the wave number of the radiation (**Figure 4.3**).

Usually, to analyse the sample surface, FTIR is coupled to attenuated total reflectance (ATR) correction, whereby infrared light passes through a crystal and penetrates the sample by only a few micrometres. In the FTIR transmission mode, the infrared light passes through the sample to reveal its bulk properties (Tiquia-Arashiro et al., 2023). In **Paper 5**, ATR-FTIR was applied to evaluate the surface of lignin after hydrolysis with or without laccase. Laccases form carbonyl groups at the benzylic position in the presence of oxygen (Agustin et al., 2021). Therefore, the region of interest was between 1500 and 2000 cm^{-1}, coinciding with where double bonds are detected.

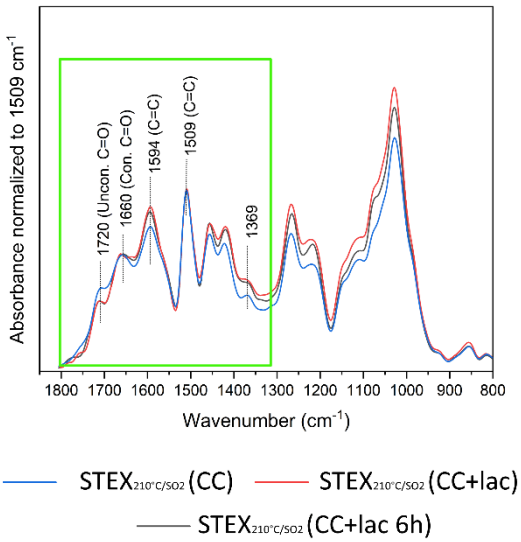

Figure 4.3. ATR-FTIR spectra of residual STEX$_{210°C/SO2}$ after enzymatic hydrolysis. The analysis was performed on residual material after 72 h of enzymatic hydrolysis under three conditions: Celluclast® + Novozym® 188; Celluclast® + Novozym® 188 + *Cc*Lcc9; and Celluclast® + Novozym® 188 + *Cc*Lcc9 (6 h pre-incubation). The green box denotes the spectral region discussed in the text (**Paper 5**).

Chapter 5. Enzymatic saccharification

In 2023, the enzymes market was worth 7.42 billion USD, and it is expected to reach 11.42 billion USD by 2030 (Report Coverage & Deliverables Market Dynamics, 2023). Amylases, proteases, and lipases are widely used in the food processing and detergent industries; however, new enzymes are continuously being discovered to facilitate other industrial processes (Sakhuja et al., 2021). A growing market includes enzymes capable of lignocellulosic biomass saccharification. Several of them are isolated and characterized from microorganisms that grow on wood or other plant substrates. Enzymes active on glycoconjugates, oligo- and polysaccharides are collectively referred to as Carbohydrate-Active enZymes (CAZymes) and classified in the Carbohydrate-Active EnZyme database (CAZy, "http://www.cazy.org") (Cantarel et al., 2009). Companies have used some of these enzymes to develop cocktails which contain all, or most, of the enzymes needed for lignocellulose deconstruction. It is necessary to consider that saccharification efficiency is affected not only by enzyme-dependent parameters, but also by biomass structure (Alvira et al., 2010). As discussed in Section 1.2 and reported elsewhere (Adsul et al., 2020; Du et al., 2020), glucose release efficiency by one mixture like Cellic® CTec2 depends on the target substrate.

This chapter begins with the definition of the base case for enzymatic hydrolysis, followed by a description of the enzymes (alone or in mixtures) that can complement the cellulolytic cocktail. Furthermore, biomass structure before and after enzymatic saccharification (**Papers 2–5**) is compared and combined with enzymatic activity to aid the development of tailor-made cocktails.

5.1 Enzymatic machinery for biomass degradation

The complex structure and composition of carbohydrates in lignocellulosic biomass is reflected in the panel of enzymes involved in its breakdown, including cellulases, hemicellulases, and lignin-active enzymes (Dedes et al., 2020) (**Figure 5.1**).

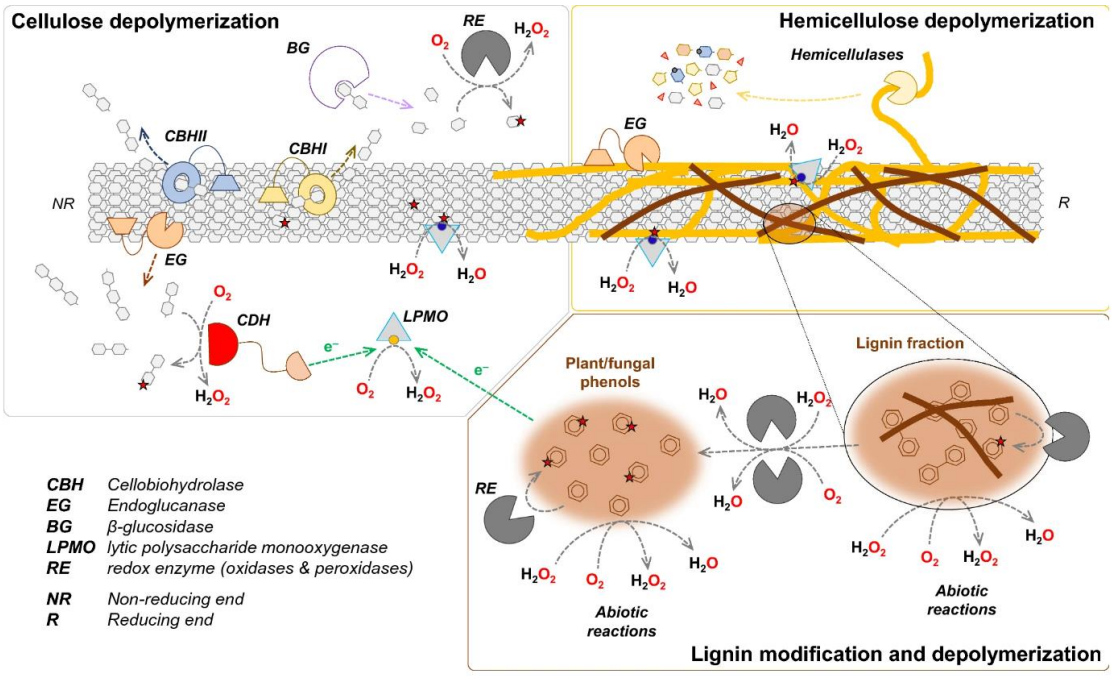

Figure 5.1 Schematic representation of the main enzymes involved in the depolymerization of a cellulose microfibril covered with hemicellulose (orange) and lignin (brown). The non-reducing (NR) and reducing (R) ends of the cellulose chains are marked. Red stars indicate oxidation catalyzed by LPMOs (triangles) or other redox enzymes (RE, grey). Interactions between hydrolytic and redox enzymes are indicated. For simplicity, the multitude of hemicellulose-active enzymes, including debranching enzymes, are indicated as "hemicellulases", while lignin-active enzymes are referred to as redox enzymes ("RE"). The figure was retrieved from Østby et al., 2020 under CC BY 4.0 license.

5.1.1 Cellulases and lytic polysaccharide monooxygenases

Four classes of enzymes are crucial for the synergistic hydrolysis of cellulose: endo-glucanases, exo-glucanases (also known as cellobiohydrolases or CBHs), β-glucosidases (also known as cellobiases), and LPMOs. Glucan chains are randomly hydrolysed by non-processive endo-β-1,4-glucanases, which open new sites for attack by processive exo-β-1,4-glucanases (CBH) (Kleman-Leyer et al., 1996). These CBHs can act on the cellulose chain ends processively, without dissociating from the substrate, cleaving cellobiose units from either the reducing (CBH I) or nonreducing (CBH II) end. β-glucosidase activity is important, because cellobiose is an inhibitor for exo-β-1,4-glucanases (Kostylev & Wilson, 2012). In 2010, oxidation-dependent cellulose degradation by LPMOs was discovered. The main role of LPMOs is to create nicks in the more ordered structure of cellulose, facilitating its subsequent conversion by canonical cellulases (Vaaje-Kolstad et al., 2010). LPMOs have also been found to cleave the backbone of hemicelluloses (Tõlgo

et al., 2022). Catalysis by LPMOs requires reduction of the active-site copper from Cu(II) to Cu(I) by a reducing agent (i.e. ascorbic acid or lignin) and H_2O_2 (or O_2) as a co-substrate. The introduction of a hydroxyl group at either the C1 or C4 position by LPMOs leads to spontaneous cleavage of β-(1-4)-glycosidic bonds and formation of a lactone (C1 oxidation) or a 4-ketoaldose (C4 oxidation) (Chylenski et al., 2019; Eijsink et al., 2019; Quinlan et al., 2011). Additional redox enzymes, such as cellobiose dehydrogenases (CDH), cellooligosaccharide dehydrogenases, and glucose oxidases, are active on cellulose-derived products and may work in concert with LPMOs (Kracher et al., 2016).

Some of these enzymes might contain an additional module referred as carbohydrate-binding module (CBM), with positive or negative effects on enzyme processivity. CBMs bring the enzyme closer to the substrate, facilitating enzyme-substrate interaction; however, they can also absorb strongly to the substrate, leading to low enzymatic activity. Therefore, CBM functionality is likely substrate- and enzyme dependent (Boraston et al., 2004; Hervé et al., 2010). Non-catalytic proteins known as expansins also favour cellulose hydrolysis, as they are thought to loosen the structural order of the more ordered cellulose (Eibinger et al., 2016).

5.1.2 Hemicellulases

A variety of hemicellulases are required to degrade the heterogenous structure of hemicellulose. Hemicellulases include depolymerizing enzymes, which act on the hemicellulose backbone, and debranching enzymes, which act on substitutions on the hemicellulose backbone (Lehmann, 2011). Hemicellulose is highly heterogenous and varies according to its source material in terms of amounts and types of hemicellulose present (Section 2.2.2). Here, the focus is on spruce hemicellulose (**Table 5.1**). The core enzymes required to degrade softwood hemicellulose include endo-β-1,4-xylanases, 1,4-β-xylosidases, endo-1,4-β-mannanases, and β-1-4-mannosidases. Ancillary enzymes include α-L-arabinofuranosidase and β-glucuronidase (Bhattacharya et al., 2015; Lairson et al., 2008). β-glucosidases can remove glucose units from the galactoglucomannan backbone (Yeoman et al., 2010). Notably, some LPMO9s show activity on hemicellulose (Tõlgo et al., 2023), however, their role in the hemicellulose hydrolysis is poorly studied.

Table 5.1. Main enzymes involved in galactoglucomannan and arabinoglucuronoxylan hydrolysis along with the reactions they catalyse (Bhattacharya et al., 2015; Lairson et al., 2008)**.**

Enzyme	Catalysed reaction
Endo-β-1,4-xylanases	Random cleavage of xylan backbone to release xylooligosaccharides
1,4-β-xylosidases	Release of short xylooligomers into single xylose units
Endo-1,4-β-mannanases	Random cleavage of glucomannan backbone to release mannooligosaccharides
β-1-4-mannosidases	Release of D-mannose from non-reducing ends of mannooligosaccharides
α-L-arabinofuranosidase	Release of L-arabinose from arabinoglucuronoxylan
α-glucuronidase	Release of 4-*O*-methyl D-glucuronic acid from arabinoglucuronoxylan
α-galactosidase	Release of galactose units from galactoglucomannan

5.1.3 Lignin-active enzymes

Lignin is depolymerized by oxidative enzymes found in fungi and bacteria. The main lignin-degrading enzymes are phenol oxidase (laccase) and heme-containing peroxidases (lignin peroxidase, manganese peroxidase, and versatile peroxidase) (Chan et al., 2020; Weng et al., 2021). This thesis work has focused on laccases, because previous studies demonstrated improved saccharification efficiency when hydrolysing softwood with cellulases and laccases (Moilanen et al., 2011; Palonen & Viikari, 2004).

Laccases are multicopper oxidases, which catalyse the oxidation of a phenolic substrate into a highly reactive phenoxy radical, using oxygen as electron acceptor and generating water as a by-product (Cañas & Camarero, 2010; Moilanen et al., 2011; Munk et al., 2015). The large heterogeneous polyaromatic structure of lignin allows resonance stabilization, which prolongs the half-life of these radicals (Perna et al., 2020). Laccases can also catalyse the oxidation of phenolics using a mediator. A mediator is a small compound continuously oxidized by the laccase and reduced by the substrate. Due to steric hindrance between the enzyme and the substrate, the mediator acts as a carrier of electrons between the two (Palonen & Viikari, 2004). A potential synergy between

laccases and LPMO9s has been suggested (Brenelli et al., 2018; Perna et al., 2020), with laccase-derived compounds acting as electron donors for LPMO9s. At the same time, both enzymes may compete for oxygen to catalyse their reactions (Brenelli et al., 2018). The detailed interaction between LPMOs and laccases remains to be elucidated.

5.1.4 Commercial cocktails

To hydrolyse the polymers in a given biomass, different classes of enzymes are needed. Filamentous fungi grow well on lignocellulosic biomass due to their ability to secrete CAZymes. A culture broth from filamentous fungi can hydrolyse lignocellulose, which has led to the development of enzymatic cocktails (Filiatrault-Chastel et al., 2021). An example is *Trichoderma reesei*, which was isolated during WWII in the Solomon Islands, where it was found to degrade tents and uniforms. In the following years it was discovered that, akin to many other filamentous fungi, it could secrete hydrolytic enzymes for lignocellulose degradation. This very useful ability has driven further strain improvement, leading to *T. reesei* Rut C-30 as the type strain. Among several different secreted enzymes, *T. reesei* secretes two CBHs, five endo-glucanases, and two β-glucosidases (Karagiosis, 2013). Depending on growth conditions, the *T. reesei* (Rut C-30) cellulase cocktail contains approximately 40–60% Cel7A (CBH I), 12–20% Cel6A (CBH II), 5–10% Cel7B (endoglucanase I), 1–10% Cel5A (endoglucanase II), and 1–2% β-glucosidases (Rosgaard, 2007). Its secretome contains also minor amounts of xylanases, including endo-1,4-β-xylanases and β-xylosidase (Herpoël-Gimbert et al., 2008; Meyer et al., 2009). The study of its secretome composition has attracted interest for its elevated cellulolytic capability (Lehmann, 2011).

When analysing a fungal secretome, it is important to keep in mind that different substrate compositions may lead to the secretion of different enzyme profiles during growth (Filiatrault-Chastel et al., 2021; Novy et al., 2019). In **Paper 4**, *Thermothielavioides terrestris* LPH172, a soft rot fungus, was grown on non-pretreated spruce, $STEX_{180°C/auto}$, $STEX_{210°C/auto}$, and $STEX_{210°C/H2SO4}$ to determine the influence of substrate pretreatment on the fungal secretome. This fungus was isolated from soil and compost in Asia (Thanh et al., 2019) and it was selected in **Paper 4** due to its thermophilic nature and availability of a sequenced and annotated genome that indicated a large potential for cellulose and hemicellulose deconstruction (Tõlgo et al., 2021). The spruce materials were chosen to introduce the fungus to spruce with diverse hemicellulose content, degree of lignin condensation, and cellulose microfibrils aggregation. Analysis of the secreted enzymes after growth on these materials revealed 198 non-intracellular proteins that were grouped into six different modules based on abundance (so called network analysis). Each module contained proteins with a similar secretion pattern on the different materials used (**Figure 5.2**). Overall, network analysis

revealed classes of enzymes with a similar expression pattern across secretomes, hinting a potentially synergistic activity or co-regulation. The abundance of enzyme classes within a module changed across materials even though they were all differently treated spruce. For example, enzymes grouped in the blue module (**Figure 5.2B**) became more abundant with increased severity of pretreatment; whereas those grouped in the turquoise module (**Figure 5.2C**) followed the opposite trend. These findings suggest that secretome content depends as much on the chemical composition of the substrate, as on biomass structure. Therefore it is affected by the severity of the pretreatment.

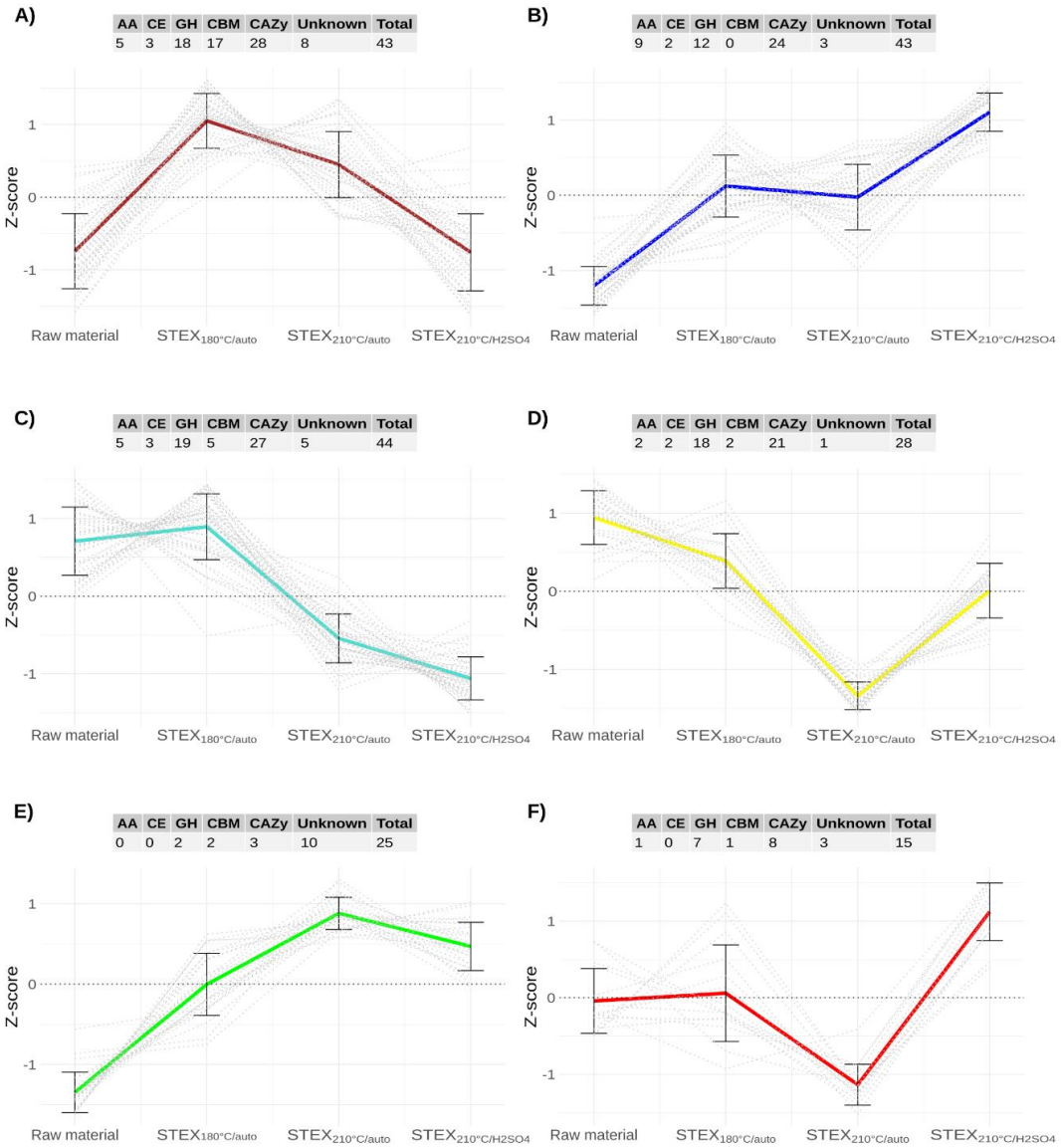

Figure 5.2. Average z-scores for protein abundance and number of proteins from the different classes of enzymes for each module. Each module corresponds to a cluster of proteins showing similar abundance across samples: (A) brown, (B) blue, (C) turquoise, (D) yellow, (E) green, and (F) red. Z-scores for each individual protein module member are plotted as dashed lines (**Paper 4**).

Depending on their formulation and target biomass, enzymatic cocktails might contain all the enzymes needed for lignocellulose saccharification; however, some cocktails may need complementary activities (Sun et al., 2015). Various companies develop enzymatic cocktails for the hydrolysis of lignocellulosic biomass. Novozymes formulated a cellulolytic cocktail using *T. reesei* (Rut C-30) named Celluclast®. Celluclast® is often supplemented with Novozym® 188, a β-glucosidase preparation from *Aspergillus niger* (Rosgaard et al., 2006), which complements the low β-glucosidases activity of Celluclast®. Cellobiose inhibits CBH and, therefore, a well-balanced β-glucosidase activity becomes important. Celluclast® contains also hemicellulolytic activity (Peciulyte et al., 2014; Lehmann, 2011).

In **Paper 1**, Celluclast® + Novozym® 188 was used to investigate enzymatic hydrolyzability of the various steam pretreated spruce samples by sugars release analysis (**Figure 5.3**). Such evaluation was important to define the base case for subsequent studies. Glucose released after hydrolysis augmented from 0% w/w DM in the raw material to 19% w/w DM in $STEX_{210°C/H2SO4}$ and 66% w/w DM in $STEX_{210°C/SO2}$ (**Figure 5.3A**). Xylose reached its highest value of 11% w/w DM in $STEX_{210°C/auto}$ (**Figure 5.3B**). Similarly, the largest release of mannose from the galactoglucomannan backbone (17% w/w DM) was detected in $STEX_{210°C/auto}$ (**Figure 5.3C**). The fact that xylose and mannose release did not increase any further in acid-catalysed steam treated materials compared to $STEX_{210°C/auto}$, indicates that the residual hemicellulose was recalcitrant to enzymatic hydrolysis. This is in line with NMR data, whereby signals for LCC were detected (**Paper 1, Table 3**). LCCs are believed to negatively affect saccharification yields because hemicellulose is covalently linked to lignin and becomes harder for enzymes to access (Raji et al., 2021; Tarasov et al., 2018).

In **Paper 2**, another commercial mixture named Ultraflo® (Novozymes) was added to the cellulolytic cocktail. According to the producer, Ultraflo® contains β-glucanase and endo-xylanase as its main activities (Rodríguez-Sanz et al., 2022); however, reports indicate the presence of minor amounts of β-xylosidase, α-L-arabinofuranosidase (Sørensen et al., 2007), cellulase (Truong & Rumpagaporn, 2019), and mannanase (**Paper 2**) activities. One of the main challenges in working with commercial cocktail is that their composition is not stated by the manufacturer. Consequently, it can be hard to draw conclusions about which enzyme activities actually contribute to the enzymatic hydrolysis.

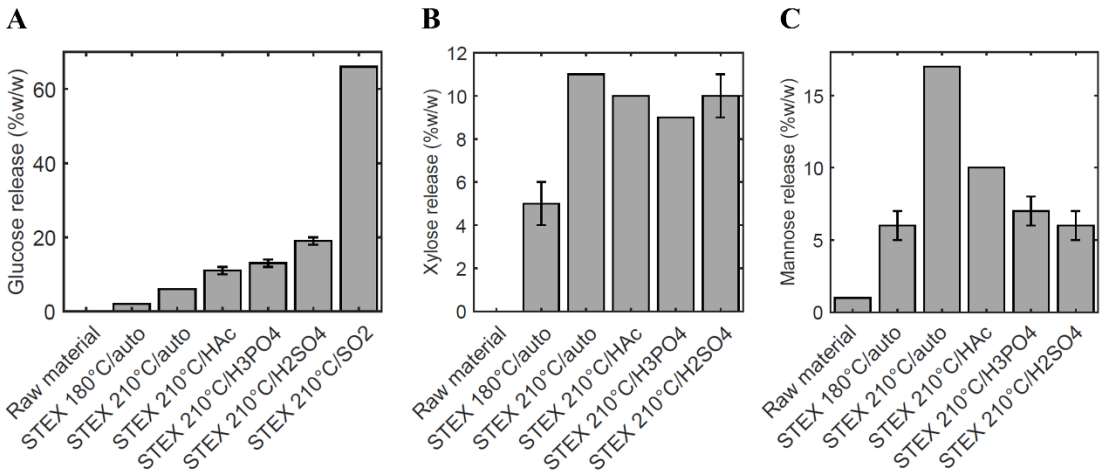

Figure 5.3. Enzymatic hydrolysis of raw and steam pretreated spruce substrates. Release of (A) glucose, (B) xylose, and (C) mannose after 48 h of hydrolysis with an enzyme loading of 10 FPU/g DM for Celluclast® and 10 U/g DM for Novozym® 188. Data represent mean values ± standard deviation of triplicate measurements (**Paper 1**).

5.2 Parameters affecting enzymatic saccharification

The low enzymatic hydrolysis efficiency of softwood represents an important hurdle for industrial-scale biorefinery applications. Owing to the complexity of lignocellulosic biomass and the need for several enzyme classes to drive saccharification, numerous parameters affect this process. They include enzyme-, substrate-, and process-related parameters (Chandra et al., 2007; Leu & Zhu, 2013; Zhao et al., 2012).

5.2.1 Enzyme-related parameters

Enzyme-related parameters affecting saccharification efficiency include synergism among enzymes involved in cellulose hydrolysis, adsorption to insoluble substrates (e.g. CBMs facilitate enzyme absorption to cellulose) (Henrissat, 1994; Mansfield et al., 1999; Teeri Tuula T., 1997), and different inhibitory mechanisms on cellulases. The understanding of the inhibitory mechanisms is relevant towards the development of efficient saccharification. Besides the inhibitory role of cellobiose on CBHs (Section 5.1.1), phenols and xylan-derived polymers inhibit cellulases in an enzyme-specific manner. Ximenes et al. determined that phenolic compounds formed during liquid

hot pretreatment (vanillin, syringaldehyde, trans-cinnamic acid, and 4-hydroxybenzoic acid) were inhibitory for cellulases and β-glucosidase. The findings show that the level of inhibition depended on the phenolic compound tested. Furthermore, inhibition levels were enzyme specific. Due to the severity of inhibition by phenolic compounds, they should be removed to ensure efficient enzymatic hydrolysis decreasing the cost efficiency of the process (Ximenes et al., 2010). Qing et al. and Zhang et al., demonstrated that xylan, xylooligomers, and xylose exerted a negative effect on cellulase and β-glucosidase activities. This was particularly evident on CBHs, when highly substituted soluble xylan was used instead of insoluble xylan (Qing et al., 2010; J. Zhang et al., 2012).

5.2.2 Substrate-related parameters

Substrate-related parameters that influence the efficiency of enzymatic hydrolysis are associated primarily with accessibility to cellulose. Accessibility can be directly related to substrate features, such as cellulose ultrastructure organization, presence of residual hemicellulose and lignin, pore size, and particle size of the material (Leu & Zhu, 2013; Mansfield et al., 1999; Novy et al., 2019; Wiman et al., 2012). Cellulose microfibrils contain regions defined by various levels of organisation, ranging from highly ordered (crystalline regions) to less ordered regions (para-crystalline regions) (see Section 2.2.1). Cellulases degrade the para-crystalline faster than the crystalline regions. Thus, it is important to determine cellulose crystallinity, because the more cellulose is ordered, the slower its hydrolysis rate will be (Novy et al., 2019; Peciulyte, 2015; Thygesen et al., 2011).

The correlation between cellulose porosity and enzyme accessibility is very important for cellulose hydrolysis, but several other structural characteristics of the biomass play a role. Lignocellulose biomass porosity increases with hemicellulose and lignin removal, impacting the enzymes accessibility to the cellulose. The more porous the surface is, the easier it is for enzymes to access the substrate. Therefore, the extent of hemicellulose and lignin solubilization or partial degradation during pretreatment, has a significant impact on the hydrolysis of pretreated material. Particle size is another key parameter affecting enzyme accessibility: the smaller is the average particle size, the larger is the surface area available for enzyme-substrate interactions. Overall, enzymatic hydrolysis is clearly connected to the structural changes induced by the selected pretreatment (Kapsokalyvas et al., 2018; Leu & Zhu, 2013; Mansfield et al., 1999; Pielhop et al., 2016).

Enzymes can unproductively bind to lignin and lower saccharification efficiency. Even if lignin is made more porous by pretreatment, unproductive binding might nevertheless diminish

saccharification efficiency. Hydrophobic interactions have been shown to contribute to non-productive binding, and cellulases bind preferentially to lignin over cellulose (Nakagame et al., 2011; Sun et al., 2016). Specifically, the aromatic component of lignin interacts with CBMs in CBM holding cellulases (Tokunaga et al., 2020). To mitigate such unproductive binding, the material can be pre-incubated with non-catalytic proteins (Liu et al., 2023). Unproductive binding of the enzymes is connected also to the S/G unit molar ratio (ratio between syringyl and guaiacyl units in the lignin), which is lower in softwood than in hardwood. The lower S/G ratio in spruce results in a more condensed lignin structure, which is more prone to unproductive binding (Liu et al., 2023).

5.2.3 Process-related parameters

In addition to being affected by the enzyme- and substrate-related parameters, saccharification efficiency is affected by process conditions, such as enzyme loading, dry mass loading (DM), temperature, and mixing (Da Silva et al., 2020; Leu & Zhu, 2013; Weiss et al., 2019). To gain insight on the effects of process parameters on hydrolysis, glucose release was measured after enzymatic hydrolysis of: $STEX_{210°C/auto}$, $STEX_{210°C/HAc}$, $STEX_{210°C/H2SO4}$, and $STEX_{210°C/SO2}$ (**Figure 5.4, unpublished data**). Generally, a higher enzyme loading leads to greater sugar release. However, this holds true only while enzyme loading is below its saturation limit; beyond that, sugar release does not increase any further (Robinson, 2015). Regardless of the amount of enzyme, the rate of hydrolysis is determined by the availability of cleavage sites. Optimum enzyme loadings depend on various factors, including the nature and composition of the substrate and the type of pretreatment method. For instance, materials with a high lignin content usually require higher enzyme loadings for complete saccharification, because a portion of enzymes will bind onto the lignin surface due to non-productive adsorption (Da Silva et al., 2020).

Three different enzyme loadings (5 FPU/g DM, 10 FPU/g DM, and 20 FPU/g DM) were tested in $STEX_{210°C/auto}$ (**Figure 5.4A**). Glucose release after 48 h increased by 2.25 folds when switching from 5 FPU/g DM to 10 FPU/g DM, but only by 1.22 fold when switching from 10 FPU/g DM to 20 FPU/g DM. Thus, 10 FPU/g DM was selected as the enzyme loading for further studies.

One way to decrease enzymatic saccharification costs is to increase the amount of biomass solids, even though conversion yields tend to decrease at elevated solid content (Weiss et al., 2019). This effect is linked to decreased mixing efficiency and mass transfer limitations, as well as water availability. An evaluation of the optimal DM loading for steam pretreated spruce was performed (**Figure 5.4B**) using Celluclast® + Novozym® 188 at 2% DM, 5% DM, and 10% DM. Glucose release from $STEX_{210°C/auto}$, $STEX_{210°C/HAc}$, and $STEX_{210°C/H2SO4}$ was not significantly affected by DM loading,

except for a significant drop at 10% DM in $STEX_{210°C/SO_2}$. Therefore, given these results, subsequent enzymatic hydrolysis was carried out with 5% DM to evaluate the effect of temperature and mixing.

It is also important to make sure that the enzymatic hydrolysis is performed at the optimal temperature range. Enzymatic hydrolysis is usually performed at 50°C to avoid contamination due to the high sugar concentrations encountered during the process (Da Silva et al., 2020; Rodrigues et al., 2015). For this thesis work, it was important to find a compromise between the optimal temperature of the commercial cocktail and that of supplemented enzymes. Enzymatic hydrolysis yielded the same sugar release at either 50°C or 40°C (**Figure 5.4C**). Consequently, 40°C was selected for enzymatic hydrolysis. Good mixing improves the diffusion of enzymes into biomass (Da Silva et al., 2020). Two different mixing conditions (25 rpm and 50 rpm) were selected to evaluate the impact on glucose release (**Figure 5.4D**). An increase in glucose release was observed when increasing the mixing for all materials. Based on these findings, the parameters selected for enzymatic hydrolysis were 5% DM, 40°C, and 50 rpm; although it should be noted that they were applied only in **Paper 5**.

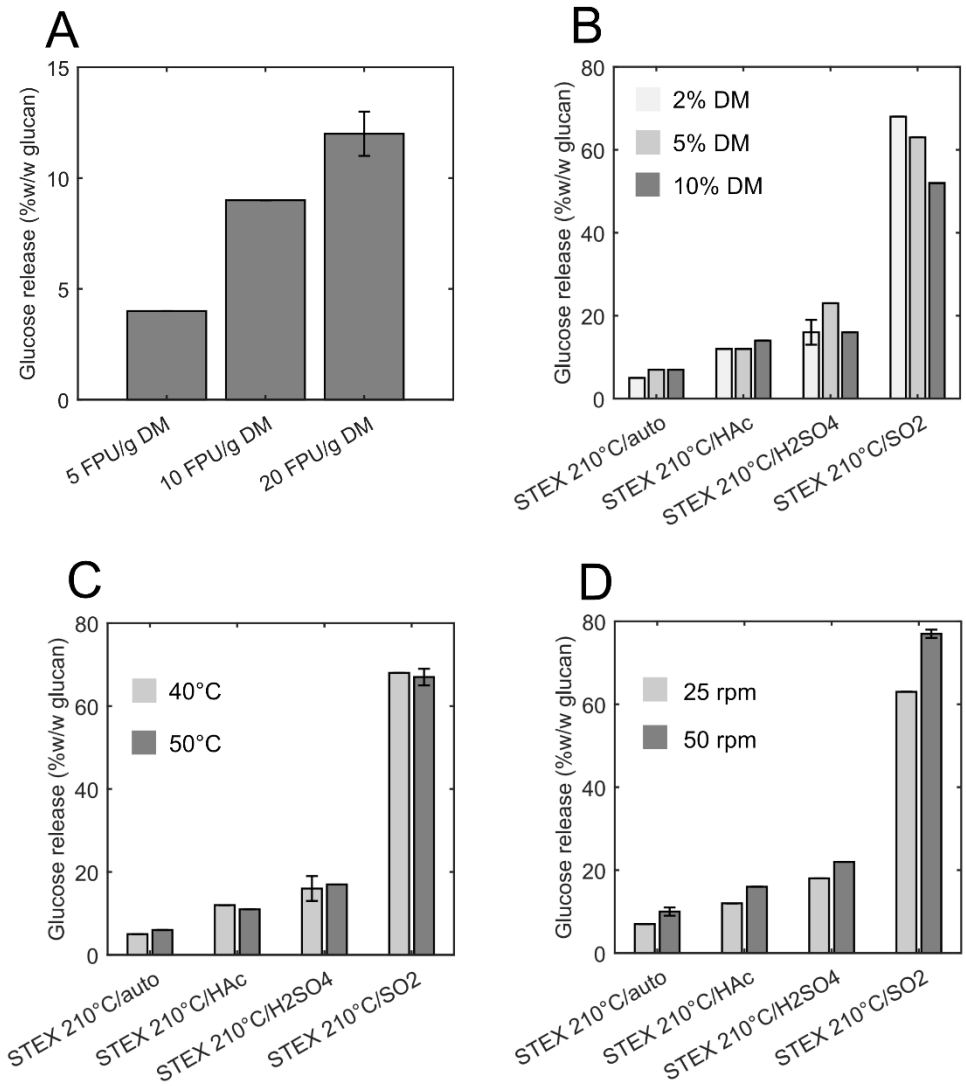

Figure 5.4. Glucose release after enzymatic hydrolysis under different conditions. (A) Glucose release from $STEX_{210°C/auto}$ after 48 h of hydrolysis with 5 FPU/g DM Celluclast® and 5 U/g DM Novozym® 188, 10 FPU/g DM Celluclast® and 10 U/g DM Novozym® 188, and 20 FPU/g DM Celluclast® and 20 U/g DM Novozym® 188. (B–D) Glucose release after 48 h of hydrolysis with 10 FPU/g DM Celluclast® and 10 U/g DM Novozym® 188 at (B) 2% DM, 5% DM, and 10% DM; (C) 40°C and 50°C; and (D) 25 rpm and 50 rpm (**unpublished data**).

5.3 Enzyme supplementation of the cellulolytic cocktail and structural evaluation of residual material after enzymatic saccharification

Typically, commercial cocktails, such as Celluclast® + Novozym® 188, exhibit low efficiency in hydrolysing biomass pretreated under mild conditions, whereby cellulose, hemicellulose, and lignin are retained in the solid fraction (Moya et al., 2024). In addition, enzyme cocktails may have different efficiencies depending on the specific composition of accessory enzymes present in the target biomass (Arnling Bååth et al., 2018; Hu et al., 2015). The varied efficiency of commercial cocktails points towards the need to adjust enzyme composition based on biomass, pretreatment method, and structural features of the pretreated materials (Oladi & Aita, 2018).

In this thesis work, the cellulolytic cocktail (Celluclast® + Novozym® 188) was supplemented with single or multiple enzymes. The effect of enzyme supplementation was evaluated not only in terms of sugar release, but also based on structural changes. Such evaluation of the residual material after saccharification was performed to evaluate the effect of the supplemented enzymes and to guide further steps in the supplementation. The enzymes supplemented to the cellulolytic cocktail were: Ultraflo®, *Thermoascus aurantiacus Ta*LPMO9A, and *Coprinopsis cinerea Cc*Lcc9 (**Papers 2, 3, and 5**).

Notably, as the different enzymes were supplemented on top of the commercial mixture, protein loading differed from that used without enzyme supplementation. Higher protein loading could increase sugar release because it might mitigate unproductive binding of enzymes to lignin (Liu et al., 2023). Thus, Celluclast® + Novozym® 188 with or without 5 g/L bovine serum albumin (as non-catalytic protein) were used for the enzymatic hydrolysis of $STEX_{210°C/auto}$, $STEX_{210°C/HAc}$, $STEX_{210°C/H2SO4}$, and $STEX_{210°C/SO2}$ to evaluate how higher protein content affected glucose release (**Figure 5.5, unpublished data**). Total protein loading did not significantly affect glucose release in the tested conditions.

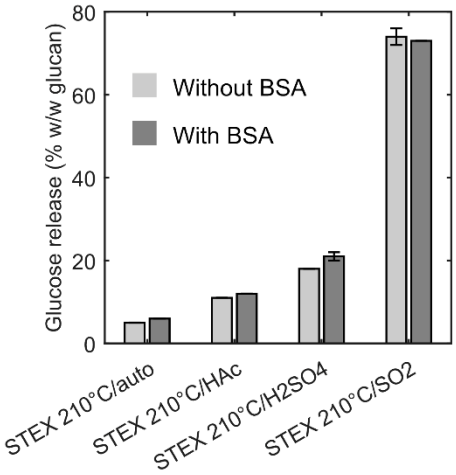

Figure 5.5 Glucose release after enzymatic hydrolysis of $STEX_{210°C/auto}$, $STEX_{210°C/HAc}$, $STEX_{210°C/H2SO4}$, and $STEX_{210°C/SO2}$. Release of glucose after 48 h of hydrolysis with 10 FPU/g DM Celluclast® and 10 U/g DM Novozym® 188 with or without 5 g/L bovine serum albumin (**unpublished data)**.

5.3.1 Ultraflo® supplementation

Considering that hemicellulose removal may increase substrate porosity (**Paper 2**), a more thorough removal should facilitate enzyme accessibility to cellulose. In **Paper 1**, hemicellulose solubilization increased with more severe pretreatment but residual hemicellulose was more recalcitrant to enzymatic hydrolysis. Therefore, Celluclast® + Novozym® 188 were supplemented with Ultraflo® to improve hemicellulose hydrolysis and evaluate the ensuing structural changes. Celluclast® is known to contain low amounts of xylanases; whereas Ultraflo® also harbours debranching enzymes, which further augment hemicellulolytic activity. Supplementation with Ultraflo® favoured the release of xylose and particularly mannose from all samples (**Figure 5.6B-C**).

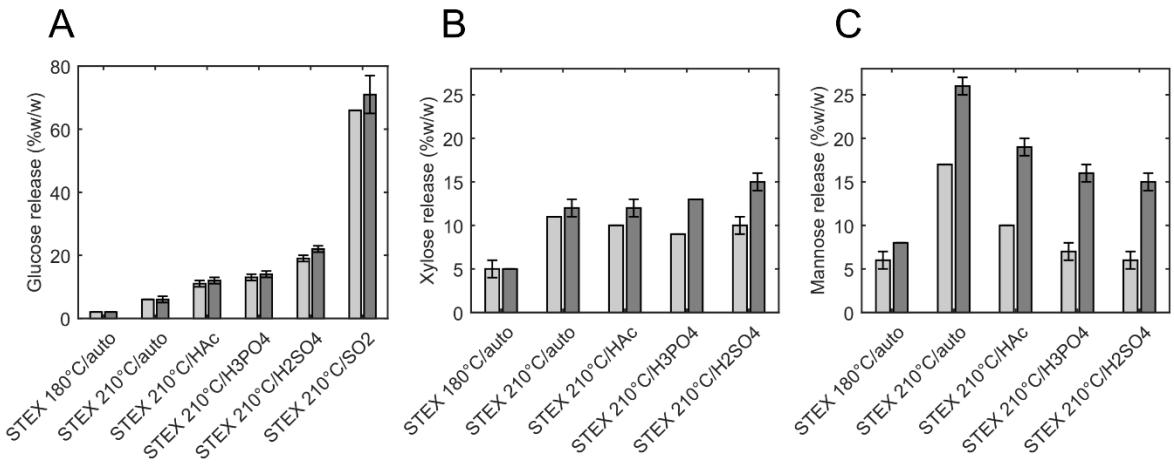

Figure 5.6 Sugar release after enzymatic hydrolysis of steam pretreated spruce with and without Ultraflo®. Release of (A) glucose, (B) xylose, and (C) mannose after 48 h of hydrolysis with 10 FPU/g DM Celluclast® and 10 U/g DM Novozym® 188 with (dark grey bars) and without (light grey bars) Ultraflo®. Ultraflo® was loaded at twice the mannanase activity measured in Celluclast® (combination of hydrolysis data from **Papers 1 and 2**).

The impact of increased hemicellulose hydrolysis was assessed by measuring the distance between cellulose microfibrils using SAXS (**Figure 5.7A**). Increased hemicellulose hydrolysis during enzymatic saccharification did not lead to further collapse of cellulose microfibrils. Consequently, there could be potential increase in the exposed surface area. The dye Congo Red binds to exposed cellulose surface areas, allowing an estimate of the exposed cellulose area to be made by measuring the amount of dye bound to the test material (Barman et al., 2020; Inglesby & Zeronian, 1996; Wiman et al., 2012). The amount of Congo Red adsorbed by the different steam pretreated materials after enzymatic hydrolysis in the presence or absence of Ultraflo® was measured (**Figure 5.7B**). Supplementation with the hemicellulolytic cocktail led to more exposed surface in the two autocatalysed materials ($STEX_{180°C/auto}$ and $STEX_{210°C/auto}$) compared to acid catalysis and can be related to more residual hemicellulose in the auto catalysed materials.

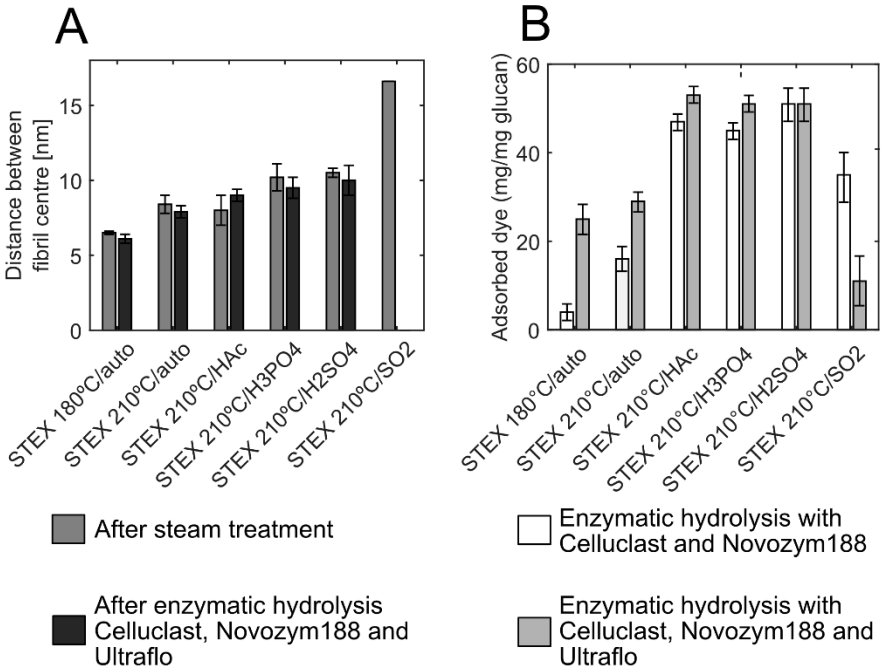

Figure 5.7. Cellulose ultrastructure evaluation before and after enzymatic hydrolysis with or without Ultraflo® supplementation. (A) The distance between cellulose fibril centres in steam pretreated materials before and after enzymatic hydrolysis with Celluclast®, Novozym® 188, and Ultraflo® was assessed using SAXS. (B) Adsorbed Congo red in the different steam pretreated materials after enzymatic hydrolysis with or without Ultraflo® (**Paper 2**).

5.3.2 *Ta*LPMO9A supplementation

LPMOs aid cellulose saccharification by creating nicks in the glucan chain on the more ordered (crystalline) parts of the cellulose microfibril (Vaaje-Kolstad et al., 2010). These nicks provide new binding sites for processive hydrolytic cellulases and, therefore, speed up the conversion rate of the enzymes on highly ordered cellulose (Novy, et al., 2019; Thygesen et al., 2011). In **Paper 1**, ultrastructural analysis revealed a slight increase in the crystallite dimension upon increased severity of pretreatment, suggesting cellulose microfibrils aggregation (**Paper 1, Figure 3**). In **Paper 3**, the contribution of the well-studied *Thermoascus aurantiacus Ta*LPMO9A to cellulose saccharification of $STEX_{210°C/auto}$, $STEX_{210°C/HAc}$, and $STEX_{210°C/SO2}$ by Celluclast® + Novozym® 188 was assessed via hydrolysis time courses. The C4 oxidized product Glc4gemGlc was qualitatively evaluated over time by HPAEC-PAD (**Figure 5.8**). *Ta*LPMO9A supplementation led to consistently higher glucose release compared to Celluclast® + Novozym® 188 alone (**Paper 3**, **Figure 3A**–**D and G**). The beneficial impact of *Ta*LPMO9A supplementation on glucose release became apparent only in the later part of hydrolysis. During enzymatic hydrolysis of $STEX_{210°C/SO2}$ (**Figure**

5.8C), C4 oxidized products accumulated for up to 24 h, after which LPMO activity likely decreased. The decrease of Glc4gemGlc could suggest that another addition of *Ta*LPMO9A at 24 h could potentially increase glucose release. Changes to cellulose crystallinity were evaluated by WAXS before and after enzymatic hydrolysis to further elucidate the role of *Ta*LPMO9A during saccharification (**Paper 3, Figure 5B**). *Ta*LPMO9A supplementation caused a decrease in crystallinity, which was exacerbated by the severity of pretreatment.

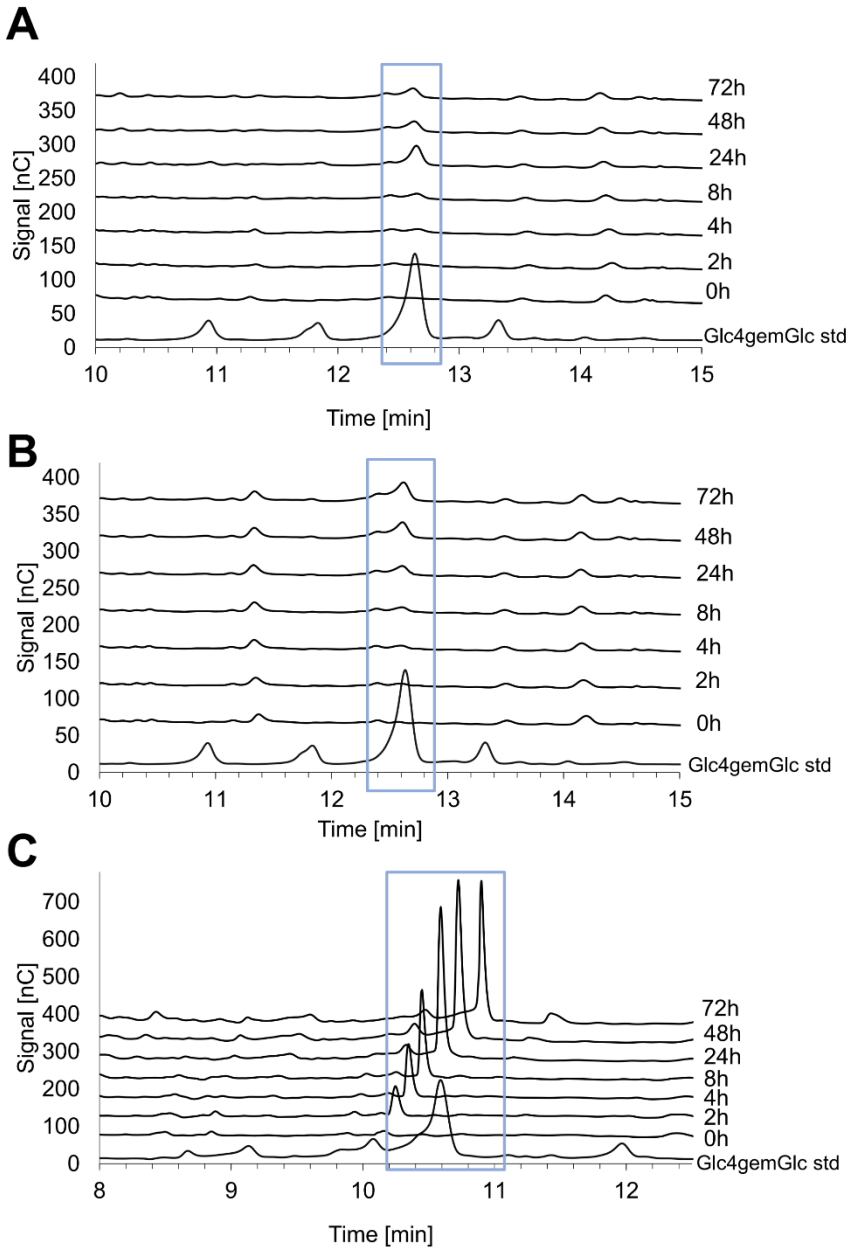

Figure 5.8. HPAEC-PAD chromatograms of the C4-oxidized glucose dimer Glc4gemGlc in time-course reactions with Celluclast® + Novozym® 188 supplemented with *Ta*LPMO9A. Chromatograms for (A) $STEX_{210°C/auto}$, (B) $STEX_{210°C/HAc}$, and (C) $STEX_{210°C/SO2}$. The Glc4gemGlc peak is enclosed by a blue box. The reactions were run in triplicates. Given the elevated similarity between all triplicate chromatograms, only one of them is presented in the figure (**Paper 3**).

5.3.3 *Cc*Lcc9 supplementation

Lignin and hemicellulose act as a physical barrier, precluding the access of enzymes to cellulose (see Section 2.2.1). In Chapter 3, the role of steam pretreatment in altering the structure of softwood has been discussed. By decreasing the severity of the pretreatment, lignin depolymerization and recondensation is diminished (**Papers 1 and 2**), resulting in a material harder to hydrolyse. Therefore, a laccase isolated from *Coprinopsis cinerea* was supplemented to the cellulolytic cocktail to further alter the lignin structure (**Paper 5**) (Kontro et al., 2021). The effect of the laccase was evaluated based on sugar release over time and structural changes to lignin at the end point by FTIR (**Figure 4.3**). Laccase was added to the reaction solution either alone for 6 h, after which the rest of the enzymes were added (pre-incubation), or simultaneously with all the enzymes. Pre-incubation with laccase was hypothesized to alter the lignin structure and expose more substrate before the addition of other enzymes. No increase in glucose release compared to the control was observed in any of the steam pretreated spruce materials ($STEX_{210°C/HAc}$, $STEX_{210°C/H3PO4}$, $STEX_{210°C/H2SO4}$, and $STEX_{210°C/SO2}$). However, analysis of the residual fraction after enzymatic hydrolysis by $STEX_{210°C/SO2}$, revealed a more condensed lignin (5-5′ linkages). Laccase oxidized the benzylic position of lignin β-*O*-4 subunits, forming conjugated ketone carbonyl structures. The lower lignin content of the other samples makes it difficult to identify these subtle changes, but they cannot be excluded. Thus, the main contribution of *Cc*Lcc9 during enzymatic hydrolysis was to induce structural changes on lignin.

5.4 Filamentous fungi secretomes as a tool for increased saccharification efficiency

In nature, filamentous fungi have a role as biomass degraders. Their ability to degrade wood components and use them as carbon sources comes from the production and secretion of CAZymes (Filiatrault-Chastel et al., 2021; Novy et al., 2019; Varriale & Ulber, 2023). Fungal wood decayers have been classified as white, brown, and soft rot fungi according to the different constituents of lignocellulosic substrates they target in nature. White rot fungi degrade extensively the lignin moiety using laccases and peroxidases before attacking cellulose. Brown rot fungi induce limited lignin modifications that allow them to gain access to cellulose and hemicellulose using both enzymatic and non-enzymatic reactions. Soft rot fungi attack plant fibers with cellulases and hemicellulases, while leaving lignin mostly intact (Goodell et al., 2008). Therefore, the study of filamentous fungi can elucidate the mechanisms of biomass degradation. Their secretome is the result of adaptation to the material on which they grow and whose recalcitrance they must overcome by secreting

appropriate hydrolytic enzymes (Peciulyte et al., 2015; Tõlgo et al., 2021). To improve saccharification efficiency, *T. reesei* cocktails have been supplemented with crude fungal secretomes. Supplementation studies based on the screening of fungal secretomes are useful tools to identify organisms whose secretomes could improve the degradation of lignocellulose when added to a commercial cocktail. The activity and composition of the best performing secretomes has hinted at the identity of the enzymes responsible for an improved performance, thus becoming ideal targets for further studies (Berrin et al., 2012; Filiatrault-Chastel et al., 2019, 2021; Peciulyte et al., 2017; Ravalason et al., 2012).

In **Paper 4**, the hydrolytic efficiency of enzymes secreted by *T. terrestris* grown on a more structurally and compositionally complex material was tested on a less complex material (**Figure 5.9**). Specifically, the enzymes secreted from the fungus after growth on $STEX_{180°C/auto}$ were chosen to test the hydrolytic efficiency on $STEX_{210°C/HAc}$, because the resulting secretome had the largest number of CAZymes. When supplementing the secreted enzymes to Celluclast® + Novozym® 188, a 2.3-fold increase in mannose release was observed. The latter can be attributed to high levels of GH5_7 in the secretome. Indeed, the secretome obtained from $STEX_{180°C/auto}$ showed the highest level of endo-β-1,4-mannanase. Increased mannan hydrolysis in $STEX_{210°C/HAc}$ is of notable importance due to recalcitrance to enzymatic hydrolysis of the residual hemicellulose.

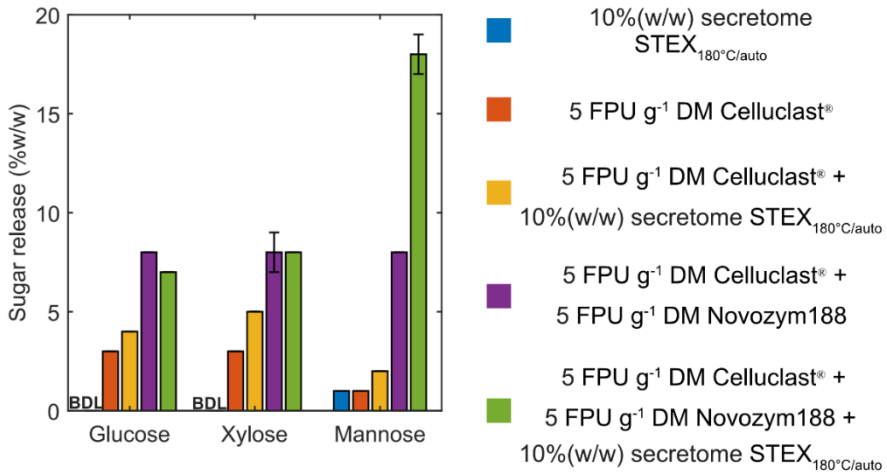

Figure 5.9. Enzymatic hydrolysis of $STEX_{210°C/HAc}$ using different combinations of commercial enzymes and the *T. terrestris* secretome. Release of glucose, xylose, and mannose was measured after 48 h of hydrolysis using: only the enzymes secreted by *T. terrestris* grown on $STEX_{180°C/auto}$, Celluclast®, Celluclast® supplemented with the enzymes secreted by *T. terrestris*, Celluclast® + Novozym® 188, and Celluclast® + Novozym® 188 supplemented with the enzymes secreted by *T. terrestris*. Data represent the mean ± standard deviation of triplicate measurements. Where error bars are not represented, they are below 0.5% (**Paper 4**).

5.5 Tailoring cellulolytic cocktails for mildly steam pretreated spruce saccharification

Even if many commercial enzyme cocktails are available, there is not a single solution that suits all hydrolysis needs. For the enzymatic hydrolysis of differently pretreated materials there is need for different enzymes profiles based on the material composition and structure (Adsul et al., 2020; Meyer et al., 2009). Ultimately, this implies the development of tailor-made cocktails step by step addition/optimisation of a base preparation with other enzymes; blending of different enzyme preparations; expression/overexpression of accessory enzymes in base-enzyme producing hosts (Adsul et al., 2020).

In this thesis work, Celluclast® + Novozym® 188 was supplemented with different enzymes preparations. This was achieved by exploiting the acquired structural knowledge and combining it with enzyme knowledge (i.e. substrate specificity or optimal rection conditions). The novelty of this approach lies in the use of material characterisation as part of selecting enzymes suitable for supplementing commercial cocktails. When developing tailor-made cocktails, it is important to use the right reaction conditions, especially if enzymes necessitate a cofactor or co-substrates. **Paper 5** sought to combine the positive effect of *Cc*Lcc9 on lignin structure with the decrease in crystallinity achieved by *Ta*LPMO9A. Phenolic compounds released by lignin-oxidizing laccases can benefit LPMOs (Brenelli et al., 2018). However, both LPMO and laccases use copper to catalyse their reactions (Eijsink et al., 2019; Munk et al., 2017), and the amount of copper should be carefully controlled to ensure optimal LPMO activity. The presence of free copper in LPMO preparations containing a reductant could lead to enzyme-independent production of H_2O_2 and enzyme inactivation (Eijsink et al., 2019; Østby et al., 2023). Given that copper can be found in softwood (Tejada et al., 2019), LPMO copper saturation should be kept under control when using the cellulolytic cocktail and a laccase. To understand whether the copper present in steam pretreated material and *Cc*Lcc9 activates *Ta*LPMO9A, sugar release was assessed for three different copper-saturated *Ta*LPMO9A preparations: 0.9 equimolar $CuSO_4$, 0.5 equimolar $CuSO_4$, and without copper. Based on saccharification efficiency and Glc4gemGlc results, *Ta*LPMO9A activity was clearly lower in the absence of copper saturation, while saccharification efficiency did not differ significantly across conditions (**Paper 5**, **Figures 3 and 4**).

The results presented in **Paper 5** show that *Ta*LPMO9A can be activated by the copper released either from the biomass or from *Cc*Lcc9. Controlling the level of free copper during enzymatic saccharification could prolong LPMO activity and prevent enzyme-independent production of H_2O_2. By altering the lignin structure, laccases could expose more cellulose for LPMOs to attach, which in turn would create more access points for canonical cellulases. Keeping LPMOs

active for longer during hydrolysis, in the presence of active laccases and canonical cellulases, could further enhance cellulose hydrolysis. These speculations need experimental verification. Laccase are known for their ability to both depolymerize and repolymerize lignin. Therefore, it should be determined if *Cc*Lcc9 solubilizes any phenols from the lignin since soluble compounds have been shown to boost LPMO activity (Brenelli et al., 2018). It is also necessary to prove that the supplementation of *Ta*LPMO9A and *Cc*Lcc9 to the cellulolytic cocktail is beneficial for saccharification efficiencies, which was not tested in **Paper 5.** In order to prove it, it is necessary to perform an enzymatic hydrolysis with and without the supplementation of *Ta*LPMO9A to the cellulolytic cocktail containing *Cc*Lcc9.

Key aspects of different steam pretreated materials:

- Supplementing targeted enzymes to a cellulolytic cocktail is beneficial for increasing saccharification efficiencies and inducing targeted structural changes

- The secretion pattern of enzymes from *T. terrestris* changed between the different spruce materials, meaning that the regulation of enzymes production is affected by the severity of the pretreatment. The resulting secretome was also beneficial as a supplement to the cellulolytic cocktail to increase mannose release.

- *Ta*LPMO9A can be supplemented together with *Cc*Lcc9 to the cellulolytic cocktail without prior copper saturation without affecting the saccharification efficiencies.

Chapter 6. Conclusions and Outlook

6.1. Conclusions

The work in this thesis investigated how to combine structural knowledge of the target material with mechanistic insights on enzyme function to build the foundation for tailor-made cocktails for the saccharification of mildly steam pretreated spruce. The low efficiency of using one single commercial mixture for the enzymatic hydrolysis of differently steam pretreated spruce is connected to the need for different enzyme profiles based on the material (**Paper 1**). The approach used to develop tailor-made cocktails was to select enzymes as supplement for the cellulolytic cocktail based on the acquired structural knowledge before and after enzymatic hydrolysis and enzymes related knowledge available in literature. The work presented in this thesis contributed to address the research questions below.

1. **What is the impact of decreasing pretreatment severity on the composition, cellulose ultrastructure, lignin condensation, and overall structure of spruce?**

By increasing the severity of pretreatment, cellulose microfibrils collapse forming microfibrils aggregates due to hemicellulose solubilization and lignin depolymerization and recondensation (**Paper 1**). These structural rearrangements result in increased surface roughness and fibrillation, contributing to greater enzyme accessibility compared to untreated spruce. Surface roughness augmented dramatically with increasing severity of pretreatment especially upon addition of acid catalyst (**Paper 2**). By combining advanced analytical techniques, it was possible to get insights on the structural characteristics of the differently pretreated spruce materials. This knowledge can guide the selection of steam pretreatment parameters to optimize the material for a specific process. Here, the aim was to obtain pretreated spruce materials with different amounts of retained hemicellulose; however, the more hemicellulose was retained, the lower was the saccharification efficiency (**Paper 1**).

2. **How can structural and enzyme knowledge be combined to optimise cellulolytic cocktails with targeted enzyme supplementation?**

Several factors affect the enzymatic saccharification efficiency of lignocellulosic feedstocks. These include enzymatic hydrolysis conditions (i.e. pH or type of buffer), enzyme-substrate interaction, material composition, and cellulose crystallinity (see Section 5.2). In this thesis, structural characteristics, such as cellulose ultrastructure, hemicellulose content, and composition, were combined with known enzyme properties to aid the selection of suitable enzyme supplements

for the cellulolytic cocktail. The analysis of saccharification efficiency and structure after enzymatic hydrolysis is fundamental in evaluating the degradation of specific structures and possible increased enzyme accessibility to the substrate.

Supplementation of the cellulolytic cocktail with *Ta*LPMO9A for saccharification, caused an increased release of glucose and xylose in $STEX_{210°C/auto}$ and $STEX_{210°C/HAc}$, along with a significant decrease in crystallinity in $STEX_{210°C/HAc}$. The accumulation of C4 oxidized products generated by *Ta*LPMO9A decreased over time, indicating reduced LPMO activity on highly ordered cellulose during hydrolysis. Therefore, adding LPMO during hydrolysis could be beneficial. (**Paper 3**).

The supplementation of Celluclast® + Novozym® 188 with Ultraflo® favoured the release of mannose and xylose, without further collapse of cellulose microfibrils (**Paper 2**). The evaluation of the exposed cellulose surface area showed a greater exposed surface in the two autocatalysed materials compared to acid-catalysed ones.

To further alter the lignin structure in less severely pretreated materials, *Cc*Lcc9 was supplemented to the cellulolytic cocktail (**Paper 5**). While this failed to boost glucose release in any of the steam pretreated spruce materials tested, it altered lignin towards a more condensed form. Thus, the main effect of *Cc*Lcc9 during enzymatic hydrolysis was on lignin.

Supplementing laccase and Ultraflo® along with Celluclast® + Novozym® 188 did not further increase the sugar release (**Paper 5**). The lack of further increase of sugars release together with the decrease in LPMO activity during enzymatic hydrolysis (**Paper 3**), highlights the importance of understanding timing supplementation with different enzymes. Enzyme addition at different time points during hydrolysis would simulate the secretion of CAZymes from filamentous fungi during growth on lignocellulosic materials (**Paper 4**).

It is important to evaluate reaction parameters to ensure that the supplemented enzymes work at their optimum. Based on the results from **Paper 5**, *Ta*LPMO9A activity was lower in the absence of copper saturation; while saccharification did not differ significantly depending on the amount of copper used for copper saturation. Keeping under control the amount of copper in the reaction solution could potentially delay enzyme inactivation.

3. Is the secretome of filamentous fungi affected by the structure of the biomass on which they grow? How can such knowledge help optimise cellulolytic cocktails?

Filamentous fungi adjust their secretome via numerous mechanisms, which can be affected by substrate structure and composition. Therefore, *T. terrestris* was grown on differently steam pretreated spruce, with similar composition, but different structural characteristics (**Paper 4**). Secretome analysis revealed classes of enzymes with similar expression patterns, whose abundance

changed depending on the material used for fungal growth. Following fungal growth on $STEX_{180°C/auto}$, the secreted enzymes were supplemented to a cellulolytic cocktail and achieved a significant increase in mannose release. Such increase was related to the secretion of high levels of GH5_7 when *T. terrestris* was grown on $STEX_{180°C/auto}$.

Concluding remarks. Trying to develop a single enzyme cocktail for the efficient hydrolysis of different materials is very challenging, as each substrate requires a distinct set of enzymes. By combining knowledge of biomass structure before and after pretreatment, with detailed enzyme knowledge, this thesis provides an innovative approach for developing tailor-made cocktails. Additionally, the work underscores the importance of evaluating reaction conditions and the design of a new enzymatic hydrolysis approach based on the addition of different enzymes during saccharification, simulating what filamentous fungi do to degrade lignocellulose in their natural environment.

6.2. Outlook

The findings presented in this thesis highlight the complexity and importance of developing tailored enzyme cocktails for the efficient saccharification of lignocellulosic biomass. Combining knowledge about biomass structure before and after enzymatic hydrolysis with that about enzyme function can lead to improvements in the saccharification efficiency. Although the improvements shown here in terms of sugar release were not industrially relevant, this approach can be further developed.

1. The first approach would involve pretreatment. In this thesis, a batch set-up with or without acid catalysts was employed. It would be interesting to try a continuous reactor, whereby spruce is pretreated at 210°C (autocatalysis) for different residence times. In a continuous reactor, parameters are better controlled, which reflects in a more effective and accurate pretreatment while using less energy. Also, a continuous set-up could be more industrially feasible from an economic point of view (Hoang et al., 2023).

2. Structural evaluation after steam pretreatment could also include measurement of the material's porosity, especially on the internal surface area. These parameters are linked to enzyme accessibility and consequently an evaluation of the cellulose accessibility could also be made with different methods to support the measure of the internal surface area (Leu & Zhu, 2013).

3. Testing the addition of enzymes at different time points during hydrolysis would simulate the way filamentous fungi hydrolyse biomass in nature (Novy et al., 2021). The time course hydrolysis with and without *Ta*LPMO9A presented in **Paper 3** shows a decline in LPMO activity around 24 h. It might be worth splitting the total LPMO loading between two time points: once at the start of enzymatic hydrolysis and once at 24 h. The same two-time loading could be applied also to the cellulolytic cocktail, as addition of only LPMO at 24 h could have no effect due to lack of fresh CBHs. A second addition of LPMOs could create new access points but, in the absence of active CBHs, cellulose hydrolysis could not proceed. A similar rationale can be extended to other classes of enzymes such as hemicellulases. These should be added not as part of the Ultraflo® mixture, but as pure enzymes, to obtain clearer results.

4. A stochastic model could be built to simulate saccharification efficiency combining structural characteristics of spruce before and after enzymatic hydrolysis, structural characterization of spruce and enzyme knowledge (e.g. substrate, kinetic parameters, and optimal conditions) available in the literature, and experimental data on enzymatic hydrolysis (Behle & Raguin, 2021). Such model would enable the simulation of sugar release upon application of either different process parameters or supplementation with different enzymes.

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