



UMEÅ UNIVERSITY

Investigations of the Importance of the Redox Environment in LPMO- Supported Bioconversion of Pretreated Lignocellulose

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Everything should be made as simple as possible, but no simpler.

-Albert Einstein

To my family

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Abstract

Achieving high yields in enzymatic saccharification of cellulose is a critical step in biochemical conversion of pretreated lignocellulosic biomass. Sugar formed during saccharification serves as substrate for fermenting microorganisms producing bio-based fuels and chemicals. An oxidoreductase, lytic polysaccharide monooxygenase (LPMO), has recently gained attention for its potential to act synergistically with conventional hydrolytic enzymes catalyzing the deconstruction of cellulose. This investigation has focused on LPMO-supported enzymatic saccharification of cellulose, exploring the process conditions, particularly the redox environment, affecting LPMO-supported saccharification of biomass. The involvement of LPMO necessitates reevaluation of industrial process configurations, especially in terms of aeration strategies. The impact of aeration on saccharification and fermentation, for example through potential side effects on fermentation inhibitors generated during the pretreatment, is not well understood, and the aim of the investigations has been to shed light on that gap of knowledge.

The role of lignin as a reductant in LPMO-supported enzymatic saccharification was investigated, focusing on both lignin in the solid fraction and water-soluble lignin degradation products in the liquid fraction. A novel experimental set-up with controlled gas addition (six parallel reactions, three with air and three with N₂) was used to regulate the redox environment. Glucose production was consistently higher in reactions with air. Both lignin in the solid fraction and degradation products in the liquid fraction efficiently supported LPMO catalysis.

The benefits of continuous aeration in LPMO-supported enzymatic saccharification were weighed against the negative effects associated with high solids loadings in reaction mixtures. Studies in the range 12.5%

to 17.5% water-insoluble solids (WIS) showed that the positive effects of aeration to support LPMO were larger than the negative effects of high solids loadings. Notably, glucan conversion with aeration at 17.5% WIS exceeded that obtained with N₂ at 12.5% WIS. Additionally, doubling the enzyme dosage was less effective in enhancing glucan conversion than using aeration rather than N₂. These findings demonstrate the significant potential of continuous aeration to boost LPMO activity when using high solids loadings in biomass conversion.

A hybrid hydrolysis and fermentation (HHF) process, incorporating an initial pre-hydrolysis phase with aeration at a relatively high temperature, was compared to simultaneous saccharification and fermentation (SSF). Using steam-exploded softwood as substrate, pre-hydrolysis with aeration improved glucan conversion in HHF, but the overall conversion remained modest. Extending the aeration period from 24 h to 48 h slightly enhanced saccharification but had a negative impact on the subsequent fermentation with *Saccharomyces cerevisiae* yeast. Thus, under the experimental conditions used, HHF with aeration led to increased glucan conversion, but the benefits were not sufficient to achieve an ethanol yield and productivity that was comparable to those achieved using SSF.

The potential negative impact of aeration on subsequent fermentation was investigated further in studies of the liquid phase of steam-exploded softwood. Compared to parallel N₂ control reactions, aeration caused a more inhibitory environment for *S. cerevisiae* yeast. Although the concentrations of some inhibitors, such as furfural, decreased during aeration, there was a slight but consistent increase in the concentrations of formaldehyde, a phenomenon that could, at least partially, explain increased inhibition. Sulfite detoxification was effective regardless of aeration. Laccase treatment showed mixed effects on fermentability, which could be attributed to the treatment causing an overall decrease in

the content of phenolic inhibitors, but also formation of more toxic substances from relatively harmless precursors.

Abbreviations

2D-HSQC	Two-dimensional heteronuclear-single-quantum coherence
ABE	Acetone-butanol-ethanol
ADP	Adenosine diphosphate
AFEX	Ammonia fiber explosion
ASL	Acid-soluble lignin
ATP	Adenosine triphosphate
BDP	Biorefinery Demonstration Plant
BECCS	Bioenergy carbon capture and storage
BET	Brunauer – Emmett – Teller
CBH	Cellobiohydrolase
CBM	Carbohydrate-binding module
CBP	Consolidated bioprocessing
CP-MAS	Cross-polarization magic-angle spinning
CrI	Crystallinity index
DA-HTP	Diluted-acid hydrothermal pretreatment
DB	Direct Blue
DMSO	Dimethyl sulfoxide
DNPH	2,4-Dinitrophenylhydrazine
DO	Direct Orange
DP	Degree of polymerization
EG	Endoglucanase
EJ	Exajoule
ESI	Electrospray ionization
FC	Folin-Ciocalteu's reagent
FTIR	Fourier-transform infrared
GH	Glycoside hydrolase
GHG	Greenhouse gas
HHF	Hybrid hydrolysis and fermentation
HMF	5-Hydroxymethylfurfural
HPAEC	High-performance anion-exchange chromatography
HPLC	High-performance liquid chromatography

HTC	Hydrothermal carbonization
HTL	Hydrothermal liquefaction
HTP	Hydrothermal pretreatment
HTP-SE	Hydrothermal pretreatment with steam explosion
IEA	International Energy Agency
IPCC	Intergovernmental Panel on Climate Change
IRENA	International Renewable Energy Agency
LCC	Lignin-carbohydrate complex
LPMO	Lytic polysaccharide monooxygenase
MS/MS	Tandem mass spectrometry
NADH	Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance
NREL	National Renewable Energy Laboratory
PAD	Pulsed amperometric detection
PL	Pretreatment liquid
PS	Pretreated solids
Py-GC/MS	Pyrolysis-gas chromatography/mass spectrometry
QqQ	Triple quadrupole
RID	Refractive index detector
SEM	Scanning electron microscopy
SHF	Separate hydrolysis and fermentation
SS	Soluble solids
SSF	Simultaneous saccharification and fermentation
SSL	Spent sulfite liquor
TAC	Total aromatic content
TCAC	Total carboxylic acid content
TCI	Total crystallinity index
TPC	Total phenolic content
TS	Total solids
UHPLC	Ultra-high-performance liquid chromatography
UV	Ultraviolet
VVM	Volume per reaction mixture volume per min
WIS	Water-insoluble solids
XRD	X-ray diffraction

List of publications

Paper I

Tang, C., Gandla, M. L., and Jönsson, L. J. (2022). Comparison of solid and liquid fractions of pretreated Norway spruce as reductants in LPMO-supported saccharification of cellulose. *Front. Bioeng. Biotechnol.* 10, 1071159. doi:10.3389/fbioe.2022.1071159

Paper II

Tang, C., Gandla, M. L., and Jönsson, L. J. (2023). LPMO-supported saccharification of biomass: Effects of continuous aeration of reaction mixtures with variable fractions of water-insoluble solids and cellulolytic enzymes. *Biotechnol. Biofuels Bioprod.* 16, 156. doi:10.1186/s13068-023-02407-y

Paper III

Tang, C., Cavka, A., Bui, M., and Jönsson, L. J. (2024). Comparison of simultaneous saccharification and fermentation with LPMO-supported hybrid hydrolysis and fermentation. *Front. Bioeng. Biotechnol.* 12, 1419723. doi:10.3389/fbioe.2024.1419723

Paper IV

Tang, C., Martín, C., and Jönsson, L. J. Bioconversion of pretreated softwood: Effects of aeration on inhibitors and fermentability using *Saccharomyces cerevisiae* yeast (Manuscript)

Additional publications by the author

Gandla, M. L., **Tang, C.**, Martín, C., and Jönsson, L. J. (2022). Enzymatic saccharification of lignocellulosic biomass. In: *Agricultural Biocatalysis*, Vol. 9. *Enzymes in Agriculture and Industry*. Editors: Jeschke, P. and Starikov, E. (Singapore: Jenny Stanford Publishing Pte. Ltd.), pp. 413–469.

The author's contributions are detailed in the publications.

Enkel sammanfattning på svenska

Lignocellulosa, en rikligt förekommande förnybar naturresurs som huvudsakligen består av cellulosa, hemicellulosa och lignin, har fått stor uppmärksamhet som råvara för produktion av biobaserade drivmedel, kemikalier och material. Bioraffinering av lignocellulosa och annan biomassa utgör ett förnybart alternativ till raffinering av fossila resurser som olja, kol och naturgas, vilket är av intresse av miljöskäl och för bättre energisäkerhet. Ett sätt att förädla lignocellulosa är genom biokemisk konvertering, som vanligtvis bygger på processteg som hydrotermisk förbehandling, enzymatisk försockring av råvarans polysackarider, mikrobiell fermentering av frigjorda sockerarter och vidareförädling av den fasta återstoden som huvudsakligen består av lignin.

En framgångsrik biokemisk konvertering bygger i stor utsträckning på att uppnå höga sockerutbyten från råvarans huvudsakliga polysackarid, som är cellulosa. Ett numera vanligt tillvägagångssätt är att försockra cellulosan med hjälp av enzymer. Fördelar med enzymatisk försockring inkluderar milda reaktionsbetingelser och låg biproduktbildning.

Vid enzymatisk försockring av cellulosa används traditionellt enzymer som bryter ned cellulosan i reaktioner med vatten, s.k. hydrolytisk nedbrytning. De vanligaste hydrolytiska enzymerna som katalyserar nedbrytning av cellulosa är cellobiohydrolas, endoglukanas och β -glukosidas. På senare tid har emellertid ett enzym som katalyserar redoxreaktioner med cellulosa rönt ökad uppmärksamhet. Detta enzym, oxidoreduktaset LPMO (lytiskt polysackaridmonooxygenas), finns i moderna enzympreparat för försockring av cellulosa och har potential att bidra till högre sockerutbyten.

Även om LPMO finns närvarande i de enzympreparationer som man använder för försockring av förbehandlad lignocellulosa är det inte säkert att dess fulla potential kommer till nytta. För att den ska göra det

krävs lämpliga reaktionsbetingelser, vilket bl.a. innebär närvaro av en oxidant som driver reaktionen, t.ex. syre från luften. Fokus för denna avhandling ligger just på vilka reaktionsbetingelser och processkonfigurationer som är lämpliga för att den inneboende katalytiska potentialen hos LPMO ska komma till sin nytta. Reaktionen med LPMO har studerats i liten laboratorieskala såväl som under industriiska förhållanden i en demonstrationsanläggning. Resultaten visar att redoxbetingelserna har mycket stor betydelse för utfallet av försöksreaktioner med LPMO och att åtgärder som luftning för att tillgodose den LPMO-katalyserade reaktionens behov av syre även påverkar vissa processtörande ämnen och därmed även påföljande förjäsningprocesser med jästsvampen *Saccharomyces cerevisiae*. Studier inom området är viktiga för att få bättre förståelse för underliggande reaktioner i samband med försöksring och förjäsning och för att utforma industriella processer som bygger på biokemisk konvertering på ett bättre sätt och därmed göra dem mer konkurrenskraftiga.

Introduction

Heavy reliance on fossil resources, such as oil, coal, and natural gas, has raised significant discussions regarding environmental issues and energy security. Scientific studies point towards human activities, including combustion of fossil fuels and land-use changes, that have led to the release of greenhouse gases (GHGs) which affect the climate (IPCC, 2023). Furthermore, a growing global population and an increasing energy demand have led to a rapid increase of GHG emissions. Global energy consumption growth accelerated in 2023 with around +2.2%, which can be compared to an average growth rate of around +1.5% per year from 2010 to 2019. The global primary energy demand in 2023 was 620 exajoules (EJ), which was a new record (Energy Institute, 2024). The share of fossil fuels in the energy mix was 81%. Specifically, oil accounted for 32% of the energy demand, coal for 26%, and natural gas for 23% (Energy Institute, 2024). Rapid population growth, rising energy demand, and international conflicts make energy security an urgent concern. Energy security encompasses more than just the uninterrupted supply of energy, as it also involves ensuring that energy is available at affordable prices (IRENA, 2023). Energy transition from fossil sources to sustainable energy alternatives offers a chance to build a safer and more sustainable energy system that reduces fuel price volatility. However, energy from sustainable sources has not been sufficient to meet the growing energy demand (IEA, 2022). Promoting renewable energy options, implementing energy efficiency measures, and adopting cleaner transportation technologies are important for alleviating anthropogenic climate change and reducing the reliance on fossil fuels.

Biorefinery

Biorefineries can be seen as analogous to oil refineries where crude oil is fractionated into a variety of products. Biorefinery aims at utilizing renewable feedstocks by integrating various technologies to convert them into valuable products, which offer alternatives in the transition to a more sustainable low-carbon economy. The term "biorefinery" was introduced in the early 1990s (Wyman and Goodman, 1993). Various definitions of the term "biorefinery" exist. For instance, IEA Bioenergy Task 42 has defined a biorefinery as the "sustainable processing of biomass into a spectrum of marketable biobased products (chemicals, materials) and bioenergy (fuels, power, and/or heat)" (De Jong et al., 2011). Similarly, the American National Renewable Energy Laboratory (NREL) describes a biorefinery as "a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass" (NREL, 2007). The pulp and paper industry can be considered as the first industrialized biorefinery system since other bio-based products are produced beyond pulp and paper (Alén et al., 2015; Jönsson and Nilvebrant, 2024). Examples of wood-based biorefineries include, for example, Borregaard in Sarpsborg in Norway, one of the most advanced biorefineries in operation for more than 40 years (Rødsrud et al., 2012), and, since 1990, Domsjö Fabriker in Örnsköldsvik in northern Sweden, which transitioned from traditional pulp production to become a biorefinery with products such as cellulose for textile manufacture, lignin as an additive in concrete manufacture, and cellulosic ethanol produced from spent sulfite liquor. Both these biorefineries utilize softwood as feedstock.

There are different types of processes that can be used for conversion of biomass to produce valuable products. These include mechanical, thermochemical, chemical, and biochemical processes. Thermochemical

processes involved in biomass conversion include gasification, pyrolysis and combustion. Gasification involves heating biomass at high temperatures (above 700 °C) in a low-oxygen environment to produce syngas, a mixture of hydrogen (H₂), carbon dioxide (CO₂), carbon monoxide (CO), and methane (CH₄) (Chang et al., 2023). Syngas can be used directly as biofuel or as a chemical intermediate in the production of other fuels and chemicals, such as methanol, dimethyl ether, ethanol, and isobutene (Chang et al., 2023). Pyrolysis occurs at intermediate temperatures (300-600°C) in the absence of oxygen, converting the feedstock into liquid pyrolytic oil (bio-oil), solid charcoal, and light gases similar to syngas (Meier, 2019). Hydrothermal carbonization (HTC) and Hydrothermal liquefaction (HTL) are both thermochemical processes used to convert biomass or residues from biomass processing into valuable forms of carbon or fuel. HTC involves the heating on biomass in water at relatively low temperatures (typically between 180°C and 250°C) under autogenous pressure. HTC converts organic material into a carbon-rich solid product, hydrochar. HTL (250-374°C at 10-25 MPa) usually converts wet biomass into a liquid bio-crude oil that is later refined into biofuels. Compared to HTC, relatively high temperature and pressure is required for HTL (Lachos-Perez et al., 2022). Additionally, combustion, which is the oldest and most widely used method for biomass conversion, is performed by heating biomass in an oxygen-rich environment to produce heat, steam, and electricity (Senneca et al., 2007). Combustion can also be important for the chemical recovery systems in biorefineries, such as recovery boilers used in the kraft process (Mboowa, 2021). This is a crucial aspect of the process, as chemical-intensive processing of biomass requires recycling of the chemicals. The carbon dioxide that is formed from combustion of biomass can tentatively be used to produce methanol, which is an example of Bio-CCS or BECCS (bioenergy carbon capture and storage)

(Zhang et al., 2017; Lefvert and Grönkvist, 2024). Transesterification, a widely used chemical method for biodiesel production, involves converting vegetable oils into fatty acid methyl or ethyl esters — commonly known as biodiesel — through a chemical reaction with an alcohol (Gufrana et al., 2023).

Anaerobic digestion, a form of biochemical conversion, involves the use of microorganisms to break down biodegradable organic material in the absence of oxygen. The process typically occurs in the temperature range 30 to 65 °C. The primary end-product of anaerobic digestion is biogas, which mostly consists of methane and carbon dioxide with trace amounts of other gases, such as hydrogen sulfide and ammonia. Biogas can be upgraded to contain more than 97% methane, making it a valuable substitute for natural gas (IEA Bioenergy Task 37, 2022).

With respect to mechanical refining of wood, the groundwood process is the oldest (Mboowa, 2021). It has largely been replaced by thermo-mechanical processing and chemi-thermomechanical processing. Whereas mechanical processing preserves all three major organic constituents of wood (cellulose, hemicellulose, and lignin), chemical refining of wood typically targets and dissolves the lignin. Among the methods used for chemical refining of wood, the kraft process is the most common. Other options include the sulfite process, the soda process, and the organosolv process. There are also different ways to carry out each of these processes.

Residual lignin from biorefinery processes can be valorized using thermochemical techniques (Brienza et al., 2023), such as pyrolysis and hydrothermal liquefaction, or by using chemical-catalytic cracking processes in which the lignin is degraded to low-molecular-mass substances that can be used as liquid biofuel. Such biofuels are of interest, for instance, for marine engines that are compatible with fuels that consist of complex mixtures.

Lignocellulosic feedstocks

Different forms of biomass have large potential as feedstocks for biorefining due to the vast amounts of reserves, amounting to approximately 1.8 trillion tons on land and to approximately 4 billion tons as aquatic reserves (Tursi, 2019). Biomass is typically relatively inexpensive and has a high production rate and has therefore gained a lot of attention for sustainable production of bio-based fuels, chemicals, and materials (De Bhowmick et al., 2018). Lignocellulosic biomass is commonly associated with residues from forestry (e.g., softwood from conifers and hardwood from broadleaf trees) and agriculture (e.g., wheat straw, corn stover), and agro-industrial by products (e.g., sugarcane bagasse). An overview of the main composition of different types of lignocellulosic feedstocks is provided in Table 1.

Table 1. Main components of different types of lignocellulosic feedstocks (mass fractions in percent of dry weight).^{a,b}

Biomass	Cellulose ^a	Hemicellulose ^a	Lignin	Extractives	Minerals	Reference
Norway spruce	42.7	18.6	29.5	1.2	0.1	Normark et al., 2016
Scots pine	42.7	23.5	29.5	4.5	0.2	Normark et al., 2014
Hybrid aspen	44.0	22.8	24.8	0.8	N.D.	Wang et al., 2018b
Silver birch	38.8	27.7	28.5	1.7	N.D.	Wang et al., 2018b
Wheat straw	39.8	24.4	22.8	5.1	4.7	Ilanidis et al., 2021
Sugarcane bagasse	36.9	24.5	22.0	4.7	4.5	Neves et al., 2016

^a Cellulose approximated as content of glucan. Hemicellulose approximated as content of arabinan, galactan, mannan, and xylan. ^bN.D., not determined.

The structure and composition of lignocellulosic biomass is complex and diverse. Apart from water, the three main constituents of lignocellulosic biomass are cellulose (38-50%), hemicellulose (20-35%), and lignin (10-25%) (Fig. 1) (Hu and Ragauskas, 2012).

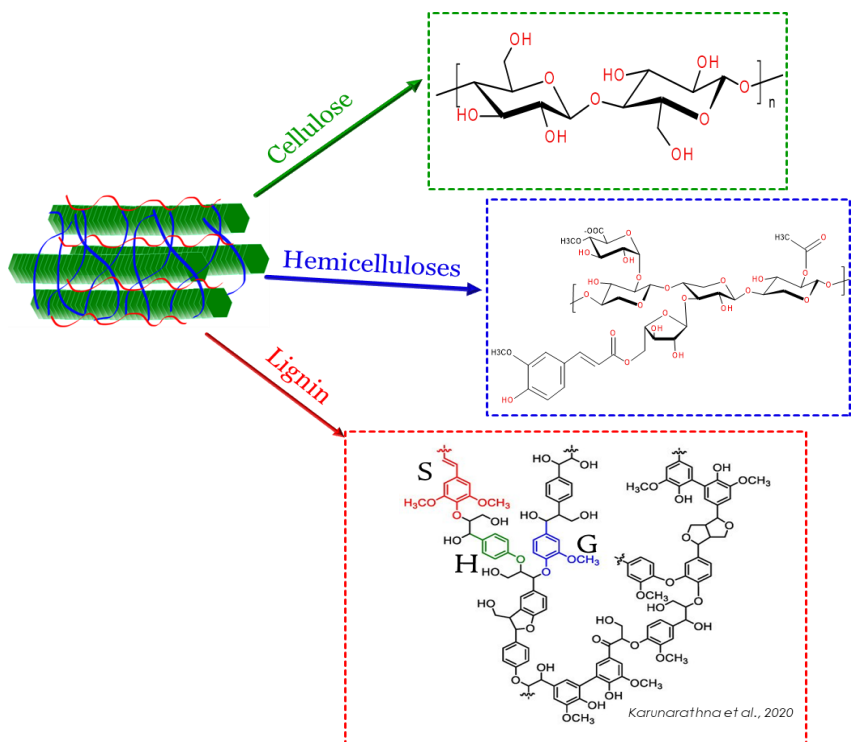


Fig. 1. The three main organic constituents of lignocellulosic biomass.

Cellulose

Cellulose is considered to be the most abundant organic substance in nature. It is a homopolysaccharide composed of β -D-glucopyranose units linked by 1,4-glycosidic bonds (Fig. 1) (Sjöström et al., 1993). It is predominantly located in the secondary cell walls.

Cellulose microfibrils in plants are formed by polymerization of glucose units into β -(1,4)-linked unbranched glucan chains. It is formed at the plasma membrane of plant cells by the cellulose synthase complex. Approximately 18-36 of these glucan chains aggregate through hydrogen bonds to form a single cellulose microfibril, but the number of chains per microfibril can vary depending on the plant (Cosgrove, 2005; Nixon et al., 2016). The orientation of cellulose microfibrils within the cell wall is crucial for its mechanical properties. There are two types of intramolecular hydrogen bonds, i.e., hydrogen bonds within individual cellulose chains (Fig. 2). One type is formed between the hydroxyl group (-OH) on the C₃ carbon atom and the oxygen atom of the neighboring glycosidic ring. The other type is formed between the oxygen atom at the C₆ position and the hydrogen atom of the C₂ carbon atom of the neighboring glucose unit. Intermolecular bonds are formed between different cellulose chains (Chen, 2014).

Cellulose macromolecules form both crystalline and amorphous regions. The crystalline regions of cellulose have a packed and ordered structure with a density of 1.588 g/cm³, whereas the density is lower in amorphous regions, i.e., 1.5 g/cm³. This is due to the wide and irregular disposition of the macromolecules in amorphous regions (Bonechi et al., 2017). The amorphous region in the cellulose structure is more accessible and is therefore more easily attacked by chemicals and cellulase enzymes. In contrast, the crystalline regions are more compact and resistant to enzymatic reactions, often requiring pretreatment to enhance their reactivity.

The degree of polymerization (DP) is a crucial structural property of cellulose, which relates to the chain length and the molecular weight, i.e., the number of glucose units. DP is defined as the number of glucopyranosyl units in the cellulose chain, and it varies in different plant species (Hallac and Ragauskas, 2011). For instance, cellulose in cotton can reach a DP of up to 10,000, while wood typically has DP values ranging from 300 to 1700 (Klemm et al., 2005; Jørgensen et al., 2007). Higher DP values mean longer chains, higher molecular weight, and fewer chain ends, features that generally contribute to the strength and rigidity of cellulose, thus also contributing to low reactivity with chemicals and enzymes (Ciolacu et al., 2011).

Over the past two centuries, cellulose has become an essential raw material for the pulp and paper industry, and for the textile fiber industry. Additionally, it plays a vital role in various fields related to renewable and sustainable energy sources (Tursi et al., 2018a; Tursi et al., 2018b; Tursi et al., 2019).

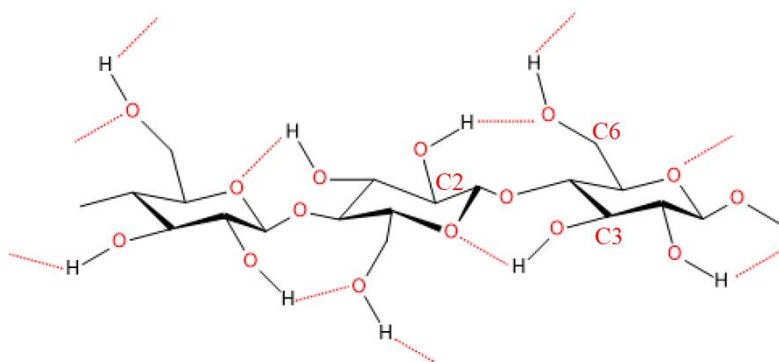


Fig. 2. Intra- and inter-molecular hydrogen bonds in the cellulose structure.

Hemicellulose

Hemicellulose is one of the major constituents of the plant cell wall. In contrast to plant cellulose, hemicellulose is a heterogeneous branched polysaccharide with a degree of polymerization of only about 200. It usually contains a variety of pentose and hexose sugar units, such as glucose, mannose, galactose, xylose, and arabinose (Sjöström et al., 1993). Hemicellulose typically accounts for 20-35% of the dry weight in wood. In softwood (i.e., wood from conifer species), hemicellulose accounts for approximately 26-33% of the dry weight, while in hardwood (i.e., wood from dicotyledonous species) it accounts for around 19-34% of the dry weight. Unlike cellulose, hemicellulose has a branched structure, is frequently acetylated, and contains uronic acid units and 4-O-methyl esters (Hu and Ragauskas, 2012). The composition of hemicellulose and the ratio of sugars units very much depends on the type of feedstock. Mannan, including water-soluble galactoglucomannan and water-insoluble glucomannan, is common in softwood. However, hardwood contains a lower fraction of glucomannan. In hardwood, xylan, including glucuronoxylan, is the predominant hemicellulose, whereas softwood contains arabinoglucuronoxylan (Donev et al., 2018). Hemicellulose is amorphous and hydrophilic and is typically easier to hydrolyze to monosaccharides than cellulose.

Lignin

Lignin is the third major biopolymer in lignocellulose. It consists of phenylpropanoid units which are linked by ether (C-O-C) and carbon-carbon (C-C) bonds to form a three-dimensional network (Fengel and Wegener, 1989, Ralph et al., 2004). The β -O-4 (β -aryl ether) linkage is the most prevalent type of interunit linkage in lignin, and it is also relatively easy to cleave (Ragauskas, 2013). Other linkages, such as β -5, β - β , 5-5, 4-O-5, are more resistant to degradation. Lignin primarily

consists of three types of phenylpropanoid units derived from three monolignols (Fig. 3): coniferyl alcohol gives rise to guaiacyl (G) units (one methoxy substituent), sinapyl alcohol gives rise to syringyl (S) units (two methoxy groups), and *p*-coumaryl alcohol gives rise to *p*-hydroxyphenyl (H) units (no methoxy substituent). Softwood lignin predominantly consists of G units, while hardwood lignin contains both G and S units with a smaller quantity of H units (Hu and Ragauskas, 2012).

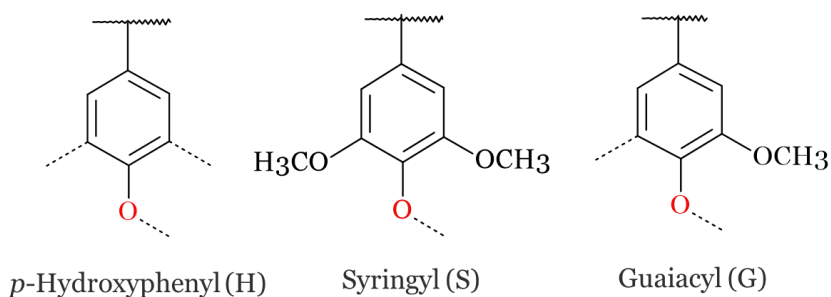


Fig. 3. Three phenylpropanoid units in lignin.

Lignin polymerization begins with enzymatic dehydrogenation of monolignols to radicals. The radicals are subsequently joined to the ends of the growing polymer through non-enzymatic combinatorial radical coupling reactions (Ralph et al., 2004).

Chemical bonds between lignin and polysaccharides (cellulose, hemicellulose) form lignin-carbohydrate complexes (LCCs) that contribute to the recalcitrance of lignocellulosic feedstocks. LCCs can be seen as hybrid structures within the plant, in which lignin is covalently linked to polysaccharides. The structures are believed to include phenyl glycoside bonds, esters, and benzyl ethers (Hu and Ragauskas, 2012).

Extractives and ash

In addition to three main organic components, lignocellulosic biomass also consists of low-molecular-weight substances, such as extractives and inorganic substances, i.e., ash (Sjöström, 1993). The composition of extractives varies depending on the feedstock. The extractives can be divided into three main groups: terpenoids and steroids, fats and waxes, and phenolic components (Marques et al., 2010). The main component of ash consists of inorganic metals, which is also feedstock-dependent and varies from less than 1% of the dry-weight of wood biomass and up to 15% dry-weight among herbaceous plants (Eom et al., 2011).

Biochemical conversion of lignocellulosic biomass

Bioconversion of lignocellulose refers to a process for production of advanced biofuels and other bio-based products (chemicals, materials) that involves one or several biocatalysts, i.e., enzymes and microorganisms (Jönsson and Nilvebrant, 2024). Other types of processes are typically also involved in bioconversion, including mechanical, thermochemical, and/or chemo-catalytic processing steps (Martín et al., 2022).

Feedstocks based on starch or sucrose have for a long time been used to produce biofuels and other bioproducts. For example, corn, with high content of starch, is the primary feedstock for bioethanol production in the US, whereas sucrose from sugarcane is the prevalent feedstock for bioethanol production in Brazil. Together, USA and Brazil produce around 80% of the world's ethanol. Beyond bioethanol, sugarcane represents one of the most successful cases of biorefinery, with three major marketable products (sugar, ethanol, and bioelectricity, as well as a great diversity of by-products (Vandenberghe et al., 2022). Compared

to feedstocks like starch and sucrose, feedstocks consisting of lignocellulosic biomass bring more challenges into the conversion due to their more complex and rigid structure. This makes it harder to use enzymes and microorganisms in the conversion process. It is therefore essential to consider pretreatment steps to make cellulose more accessible to cellulolytic enzymes before the enzymatic saccharification (Galbe and Wallberg, 2019; Martín et al., 2022). Also, the conversion of sugars through microbial fermentation needs to be followed by valorization of the lignin-rich residue (also called "hydrolysis lignin"). Fig. 4 schematically illustrates such a process.

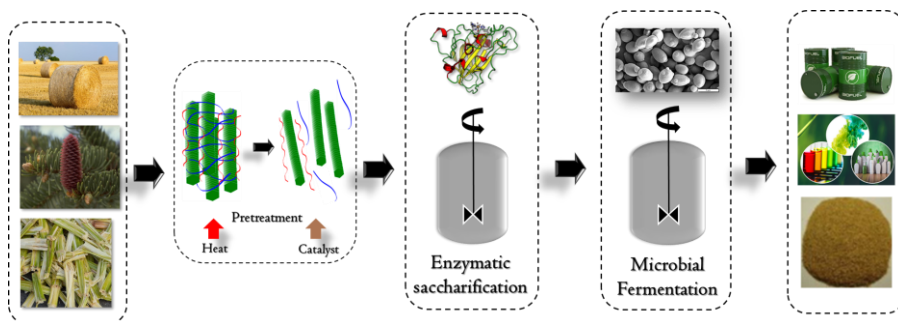


Fig. 4. Biochemical conversion of lignocellulosic biomass into value-added products.

Pretreatment

Pretreatment, or fractionation, is a crucial step in the conversion of lignocellulosic biomass, making the biomass more accessible to the enzyme, thus contributing to more efficient saccharification of the biomass. An effective pretreatment targeting hemicelluloses would result in a great performance with respect to enzymatic digestibility and recovery of hemicelluloses (Martín, et al., 2022). Pretreatment typically consists of both mechanical and hydrothermal steps.

Mechanical processing is typically applied in the beginning in order to prepare the biomass for subsequent conversion without altering its chemical composition. It includes size reduction (e.g., wood chipping, grinding, milling, shredding), separation of feedstock components (e.g., debarking, sieving, centrifugation) and physical pretreatment (e.g., pelletization, extrusion).

Depending on the chemicals that are involved, the target component, and the conditions, pretreatment methods can be divided into different groups, for instance, mild alkaline pretreatment, chemical pulping processing, hydrothermal pretreatment, oxidative pretreatment, acid-based pretreatment, or pretreatment using alternative solvents, such as ionic liquids (Jönsson and Martin, 2016).

Alkaline pretreatments typically target the lignin, and break the bonds between lignins and carbohydrates, resulting in partial solubilization of lignin. Alkaline pretreatment typically leads to less solubilization of hemicellulose and less formation of inhibitory pretreatment by-products. Different types of alkali that can be used in alkaline pretreatment include sodium hydroxide, potassium hydroxide, and ammonium hydroxide (Galbe and Zacchi, 2012). Sodium hydroxide and potassium hydroxide are most commonly used. Other alternatives for alkaline pretreatment include calcium hydroxide and ammonia. Ammonia is used for pretreatment at elevated temperatures in ammonia recycle percolation and ammonia fiber explosion (AFEX) (Chundawat et al., 2020). In AFEX, biomass is typically treated with liquid ammonia at moderate temperatures (60-100 °C) and high pressure. After the biomass has been saturated with ammonia, the pressure is released. The release of the pressure causes ammonia to vaporize and an explosion occurs. The explosion can disrupt cellulose and hemicellulose, contributing to breaking down the lignin-carbohydrate complex, and thus improving the accessibility of enzymes to cellulose and hemicellulose (Balan et al.,

2009). However, due to the rather limited effect on the biomass, mild alkaline methods, such as AFEX, are used mainly for herbaceous biomass rather than for wood.

Chemical pulping processes mainly target lignin, but to some extent also hemicelluloses (Jönsson and Martin, 2016). They are primarily used in the pulp and paper industry, to improve the efficiency and quality of paper production (Mboowa, 2021). The main process types are kraft pulping, sulfite pulping, soda pulping, and organosolv pulping, although the soda pulping was mainly used in the past and organosolv pulping has so far not been implemented extensively in the industry.

Kraft pulping involves the use of sodium hydroxide (NaOH) and sodium sulfide (Na₂S). Lignin and parts of hemicellulose are degraded into black liquor, which is used in recovery boilers for energy purposes in a process that is also a part of the system for recycling of chemicals (Mboowa, 2021). In the sulfite pulping process, bisulfite (HSO₃⁻) and sulfite (SO₃²⁻) are used to convert lignin to lignosulfonates and to hydrolyze hemicellulose into a water-soluble mixture called spent sulfite liquor (SSL). The structure of cellulose is, however, maintained. SSL from softwood mainly contains hexoses, which can relatively easily be fermented by *Saccharomyces cerevisiae* yeast into ethanol. SSL produced from hardwood is not that useful for further processing through fermentation, because most sugars are pentoses that are more difficult to ferment (Pereira et al., 2012). A modification of sulfite pulping called "sulfite pretreatment to overcome recalcitrance of lignocellulose" or "SPORL" consists of a short chemical treatment of the feedstock followed by mechanical size reduction (Shuai et al., 2010). For example, biomass is first treated with sulfite salt at 160-180 °C at pH 2-4 for 30 min, and is then fiberized (a size reduction) to generate a fibrous substrate for subsequent saccharification and fermentation (Shuai et al., 2010). SPORL produces digestible substrates with high recovery of

hemicellulose and low amounts of fermentation inhibitors. Another sulfite-based method, developed by Borregaard, is called the BALI™ process. As it is similar to the sulfite process, lignosulfonates are generated as a by-product and the cellulose in the resulting pulp has good enzymatic digestibility (Rødsrud et al., 2012). In the soda process, sodium hydroxide is used as pulping chemical. It breaks down and solubilizes lignin. It is mainly used for non-woody plants with higher content of inorganic material than wood. Whereas the kraft process, the sulfite process, and the soda process efficiently remove lignin and result in a cellulosic material, a pulp, that is relatively easy to degrade enzymatically, these are chemical-intensive processes. It is therefore essential to have effective systems for the recovery of chemicals, and the methods are typically associated with high capital expenditure costs.

Organosolv pulping is based on the use of organic solvents (such as ethanol) which are used to dissolve lignin (Pan et al., 2006). With acid catalyst, the process can be performed at lower temperatures. However, the solvent needs to be removed after the pretreatment to avoid inhibition and for recycling of process chemicals. The cost for removing the solvent is important to consider, and cost issues have prevented the use of the organosolv process in industry (Mielenz, 2020). Also, due to the volatility and flammability of the solvents, there is an interest in exploring non-volatile alternative solvents (Martín et al., 2013).

Hydrothermal pretreatment (HTP) is a mature pretreatment method for lignocellulosic biomass (Galbe and Wallberg, 2019; Ruiz et al., 2013; Martín et al., 2022). Performed in the simplest way, the HTP process involves biomass and water that is heated to temperatures in the range 150-230 °C. The process can be performed continuously and with high solids loadings, making it well suited for industrial applications (Martín, et al., 2022). The temperature and residence time used for the HTP process is adjusted to the desired severity, which is different depending

on the feedstock (typically low severity for herbaceous feedstocks and high severity for softwood) (Galbe and Wallberg, 2019). The severity factor can be calculated as a function of time and temperature (Chornet and Overend, 2017; Ruiz et al., 2021). HTP pretreatment can potentially be performed also by using a steam explosion step or by addition of acid. When hydrothermal pretreatment is combined with steam explosion (HTP-SE), the pretreated biomass is subjected to a sudden decompression resulting in a disruption of the fiber structure and further breakdown of the biomass. Addition of low concentrations of an acid catalyst, such as sulfuric acid, is sometimes referred to as dilute-acid pretreatment (DA-HTP). Regardless of whether small amounts of acid are added from an external source [such as addition of sulfuric acid, sulfur dioxide (which forms sulfurous acid), and phosphoric acid], the pretreatment chemistry will be acidic. That is because of auto-catalysis. When the breakdown of hemicellulose begins, there will be formation of carboxylic acids. These include, among others, acetic acid formed from acetyl groups in hemicelluloses, and levulinic acid and formic acid formed as breakdown products from monosaccharides and furan aldehydes. Thus, addition of small amounts of an external acid enhances the acidic pretreatment chemistry, and the resulting slurries after HTP, HTP-SE, and DA-HTP are all acidic, often with a pH in the range 1-3. Due to the acidic pH, it is the hemicellulose that is mainly affected by HTP, whereas lignin and cellulose mainly remains in the solid phase (Galbe and Wallberg, 2019; Martín et al., 2022). The severity factor can be extended to a combined severity factor, which also covers acidity (Ruiz et al., 2021). Due to high acidity, especially for DA-HTP, corrosion-resistant equipment is needed and there is typically a rather extensive formation of by-products that in sufficiently high concentrations can inhibit the fermenting microorganism. However, due to the effectiveness, rather limited capital expenditures and ease of implementation, HTP,

HTP-SE, and DA-HTP are generally good options for upscaling of biomass conversion processes, which is reflected by a relatively large number of commercialization attempts and demonstration-scale projects (Alvira et al., 2010, Jönsson and Martin, 2016; Martín et al., 2022).

Oxidative pretreatment includes alkaline peroxide pretreatment, ozonolysis, and wet oxidation. These methods typically result in a reduction of the crystallinity of cellulose and a breakdown of linkages between carbohydrates and lignin (Jönsson and Martin, 2016).

By-product formation

During the pretreatment, the operational conditions are optimized to enhance the enzymatic digestibility of the cellulose, primarily by removal of hemicellulose and/or lignin from the structure. However, degradation and solubilization of fragments of hemicelluloses, cellulose, and lignin results not only in formation of water-soluble saccharides but also in formation of undesired by-products. By-products may inhibit subsequent process steps, i.e., enzymatic saccharification and microbial fermentation (Jönsson and Martín, 2016).

Inhibition of cellulolytic enzymes can be caused by both soluble carbohydrates, such as monosaccharides, and some aromatic substances, such as phenolics, that were generated during the pretreatment. Monosaccharides and disaccharides inhibit enzymatic saccharification, which may be referred to as end-product inhibition. Hemicellulose-derived oligosaccharides may contribute to inhibition as well. Kumar and Wyman (2014) reported that manno-oligosaccharides have inhibitory effects on cellulases, and Kim et al. (2011) reported that xylo-oligosaccharides are inhibitors. Oligosaccharides can be generated in the pretreatment or as intermediates in enzymatic saccharification reactions, as cellulolytic enzyme preparations typically also contain enzymes that

deconstruct hemicelluloses. Besides soluble sugars, aromatics, mainly phenolic compounds, have been shown to have an inhibitory effect on enzymatic saccharification (Ximenes et al., 2011; Malgas et al., 2016). Ximenes et al. (2011) reported that 4 mg of vanillin can reduce the hydrolysis rate of 1 mg of endo- and exo-cellulases by 50%. In the studies of Malgas et al. (2016), lignin derivatives caused stronger inhibition of manolytic enzymes than organic acids, furans, and monosaccharides. However, inhibitory effects on enzymes caused by aromatic compounds can be alleviated by addition of certain reducing agents, notably sulfur oxyanions such as sulfite (Alriksson et al., 2011; Cavka et al., 2011; Cavka and Jönsson, 2013).

Microorganisms are inhibited by various compounds formed during biomass pretreatment, including aromatic compounds, furan aldehydes, and aliphatic acids (Jönsson et al., 2013). Aromatic compounds are generated primarily through partial degradation of lignin, which involves the cleavage of β -O-4 linkages and other linkages, and from extractives containing phenolic groups (Jönsson and Martín, 2016). The inhibitory effects of aromatic compounds are complex and difficult to attribute to a single source, as they contain a wide range of molecules with varying functional groups that contribute to microbial inhibition (Larsson et al., 2000). However, certain compounds have been identified as having particularly strong inhibitory effects on yeast growth. For instance, benzoquinone is highly toxic to yeast even at low concentrations (Stagge et al., 2015). As little as 0.19 mM of benzoquinone can completely inhibit yeast growth (Larsson et al., 2000). Furthermore, studies have shown that the inhibitory effect of formaldehyde on yeast begins already at 1 mM (Cavka et al., 2015a).

Furan aldehydes, such as furfural and 5-hydroxymethylfurfural (HMF), are primarily generated from the degradation of hemicellulose during pretreatment of biomass. These compounds are formed through

the dehydration of pentoses (producing furfural) and hexoses (producing HMF) under acidic conditions (Jönsson and Martin, 2016). The concentration of furan aldehydes in the hydrolysate varies depending on the type of biomass and the severity of the pretreatment process. The primary mechanism of inhibition is believed to be the effect on NADH regeneration, as the reduction of furfural to furfuryl alcohol consumes NADH, thereby affecting the yeast's ability to perform essential metabolic reactions (Miller et al., 2009; Jilani and Olson, 2023).

The aliphatic carboxylic acids formic acid and levulinic acid are mainly derived from the degradation of furfural and HMF. Acetic acid is released from the deacetylation of hemicellulose during the pretreatment of the biomass, or by esterases in enzyme preparations acting on residual hemicelluloses after pretreatment. When the uncharged form of the acid diffuses across the yeast cell membrane, it dissociates into its charged form and a proton, contributing to lowering the internal pH in the cell and affecting cell membrane integrity and thus inhibiting yeast growth. A total aliphatic acid concentration of around 100 mM was required to exert an inhibitory effect on yeast (Larsson et al., 1999b). However, due to low acetyl content in softwood, relatively low concentrations of acetic acid are found in softwood hydrolysates.

Besides aliphatic carboxylic acids, aromatic carboxylic acids are also found. These include, for instance, ferulic acid, 4-hydroxybenzoic acid, and cinnamic acid (Du et al., 2010). According to the study by Larsson et al. (2000), ferulic acid was inhibitory to *S. cerevisiae* even at relatively low concentrations (around 1 mM). This makes it possible that aromatic carboxylic acid contributes to inhibitory effects although they are present in much lower concentrations than aliphatic carboxylic acids. Low concentrations of carboxylic acids may have a stimulatory rather than inhibitory effect on ethanol production. This is due to the ability of the acids to disturb proton gradients across membranes and thereby

uncouple the respiratory chain and oxidative phosphorylation of ADP, leading to an increased demand for ATP, which is then satisfied by increased ethanol production (Jönsson and Martín, 2016).

Detoxification

Detoxification, sometimes referred to as conditioning, is one of the most effective strategies to overcome inhibition problems in lignocellulosic hydrolysates (Jönsson et al., 2013). Several detoxification methods have been studied. For instance, alkali treatment (Alriksson et al., 2006; Wang et al., 2024), reducing agents (Alriksson et al., 2011; Cavka and Jönsson, 2013), enzyme treatment with laccase and peroxidases (Jönsson et al., 1998; Larsson et al., 1999a; Jurado et al., 2009; Schroyen et al., 2017), polymer addition (Cannella et al., 2014), and so on.

In alkali treatment, calcium hydroxide Ca(OH)_2 , sodium hydroxide (NaOH), or ammonium hydroxide (NH_4OH) are used for treatment of hydrolysates. The pH of the hydrolysate is increased to over 9, typically around pH 12, and is maintained there for several hours, sometimes with moderate heating. However, the pH needs to be adjusted back to slightly acidic pH before performing fermentation (Alriksson et al., 2005 and 2006).

Conditioning by using reducing agents, such as sodium sulfite, sodium dithionite, and sodium borohydride, has been shown to improve the fermentability of hydrolysates (Alriksson et al., 2011; Cavka and Jönsson, 2013). These chemicals are industrial chemicals that are used in the pulp and paper industry, and they can be added directly to the bioreactor without the need for an additional process step, as in detoxification with alkali. Techno-economic analysis suggests that sulfite treatment would be a viable option. According to results by Cavka et al. (2015b), sodium sulfite conditioning could be economically justified if the yeast inoculum

concentration could be reduced by approximately 0.7 g/L (dry weight) and the enzyme dosage by 1 FPU/g of water-insoluble solids. The feasibility of this approach was confirmed through validation in demonstration scale (Cavka et al., 2015b).

Laccases are multicopper oxidase enzymes that can catalyze the oxidation of phenolic compounds, such as vanillin, coniferyl aldehyde, and syringaldehyde. Laccases utilize molecular oxygen as a substrate for oxidative reactions, and with an oxygen-rich environment, as provided by aeration, it would be more effective. Laccase reduces molecular oxygen to water and oxidizes phenolic compounds, as well as some other substrates (Upadhyay et al., 2016). This makes the laccase valuable for detoxifying by-products formed during biomass pretreatment. In a study with yeast and a phenol-rich hydrolysate from willow, laccase treatment was found to significantly improve glucose consumption and ethanol productivity (Jönsson et al., 1998).

Enzymatic saccharification

Enzymatic saccharification is used in the conversion of lignocellulosic biomass into biofuels and biochemicals since fermentable sugars are essential for subsequent microbial fermentations to produce ethanol and other bioproducts (Gandla et al., 2022; Østby et al., 2020). Cellulose and residual hemicelluloses remaining after pretreatment are broken down into oligosaccharides, disaccharides, and monosaccharides by the enzymatic treatment. Hydrolysis of cellulose using acid, such as sulfuric acid, is an option, but advantages with enzymatic saccharification include that the enzymes are highly specific for their substrates, which results in less by-product formation. Additionally, enzymatic processes generally operate under mild conditions (moderate temperature and close to neutral pH), which reduces energy consumption and the need to use equipment that is highly corrosion resistant.

The initial stages of the process can be described as morphogenesis, which refers to the dynamic changes in the structure or accessibility of cellulose substrates and enzymes during the breakdown process (Arantes and Saddler, 2010). The binding of enzymes to cellulose involves structural adaptations, for example, when the carbohydrate-binding modules (CBMs) of some enzymes assist the binding of the enzyme to the substrate (Gandla et al., 2022). This interaction can change the spatial arrangement and accessibility of cellulose fibers (Arantes and Saddler, 2010 and 2011). Many fungal cellulases and certain hemicellulases are equipped with CBMs.

Enzymatic saccharification at high solids loadings (>15% w/w) has potential to improve the economic feasibility of the process by reducing capital expenditures and operational expenditures, such as the energy demand for distillation. However, the drawback described as the "high solids effect" has shown that the sugar yield decreases when solids loadings increase, thus impacting the process efficiency and effectiveness (Humbird et al., 2010). This might be due to the higher viscosity of the pretreated material and the low water content leading to poor mixing and mass transfer, restricted enzyme diffusion, and reduced enzymatic accessibility, resulting in lower overall conversion rates (Szijártó et al., 2011). Additionally, a high sugar concentration in the reaction mixture can inhibit cellulolytic enzymes through end-product inhibition, which is also known as feedback inhibition. The concentration of fermentation inhibitors is also higher when using a more concentrated system. Overcoming these challenges might require, for instance, improving mixing techniques, using a more effective biomass pretreatment, employing adaptive bioprocessing strategies, and using enzymes with high tolerance of end-product inhibition (Da Silva et al., 2016).

Conventional cellulolytic enzymes

Conventional enzymatic saccharification of cellulose relies on hydrolytic enzymes, particularly cellobiohydrolases, endoglucanases, and β -glucosidases (Kant et al., 2024) (Fig. 5). Cellobiohydrolases primarily cleave cellobiose units from the reducing or the non-reducing ends of cellulose chains. Endoglucanases hydrolyze glycosidic bonds within the interior of cellulose chains and create new chain ends that cellobiohydrolases can act upon. The cellobiose formed by the action of cellobiohydrolases is converted into glucose by β -glucosidase (Kant et al., 2024).

A significant challenge faced by conventional cellulase enzymes during hydrolysis of lignocellulosic biomass is their limited access to cellulose due to its highly ordered fibrils. The structural features of cellulose result in that it is mainly the surface layer of the cellulose microfibrils that is accessible for enzymes, resulting in ineffective degradation of cellulose (Arantes and Saddler, 2011).

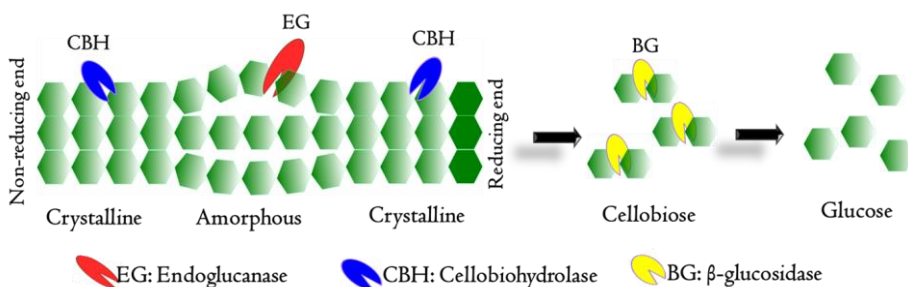


Fig. 5. Conventional model of cellulose degradation.

Lytic polysaccharide monooxygenase (LPMO)

More recently, researchers discovered an oxidoreductase enzyme that is now known as lytic polysaccharide monooxygenase (LPMO), which plays a role in the enzymatic breakdown of cellulose. Initially, LPMOs

were categorized under two families in the CAZy database: Glycoside Hydrolase Family 61 (GH61) and Carbohydrate-Binding Module Family 33 (CBM33). However, they were later named as Auxiliary Activity families AA9 and AA10 (Levasseur et al., 2013; Lombard et al., 2014). AA9 LPMOs, which originate from fungi, are active on cellulose and cello-oligosaccharides (Isaksen et al., 2014), xyloglucan (Agger et al., 2014), chitin, and xylan (Frommhagen et al., 2015). In contrast, AA10 LPMOs, which come from bacteria, primarily act on chitin and cellulose (Forsberg et al., 2014; Vaaje-Kolstad et al., 2010). Currently, LPMOs are categorized into seven families (9-11, 13-15, and 16).

The structure of LPMO exhibits an immunoglobulin-like distorted β -sandwich fold, which consists of antiparallel β -strands connected by loops and α -helices (Fig. 6) (Eijsink et al., 2019). In contrast to classic glycoside hydrolases, which have obvious substrate-binding grooves, LPMOs exhibit a relatively flat binding surface that catalyzes the degradation of cellulose. At the active site, a divalent copper atom is coordinated by three nitrogen atoms (two from side-chains of histidine residues and one from the amino group of the *N*-terminal histidine residue) forming the histidine brace, one tyrosine residue, and two water molecules (Walton et al. 2023).

Glycoside hydrolases (GHs) are a class of enzymes that catalyze the cleavage of glycosidic bonds through a hydrolysis reaction, a process that involves the participation of water molecules. The reaction results in the formation of sugar hemiacetals or hemiketals and the corresponding free aglycon (Fig. 7). In contrast to conventional hydrolytic enzymes, LPMOs cleave glycosidic bonds through an oxidative mechanism to break down cellulose chains into smaller fragments. This fragmentation enhances the activity of GHs in cellulolytic enzyme mixtures by generating additional hydrolysis targets (Hemsworth et al., 2015). LPMOs typically cleave the cellulose chain at either the C1 or C4 position of a sugar ring

(Fig. 8). Oxidation at the C1 position leads to the formation of a lactone, which then dissociates in water to produce an aldonic acid at the reducing end. Conversely, oxidation at the C4 position yields a ketoaldose at the non-reducing end, which upon hydration, forms a geminal diol (Fig. 8).

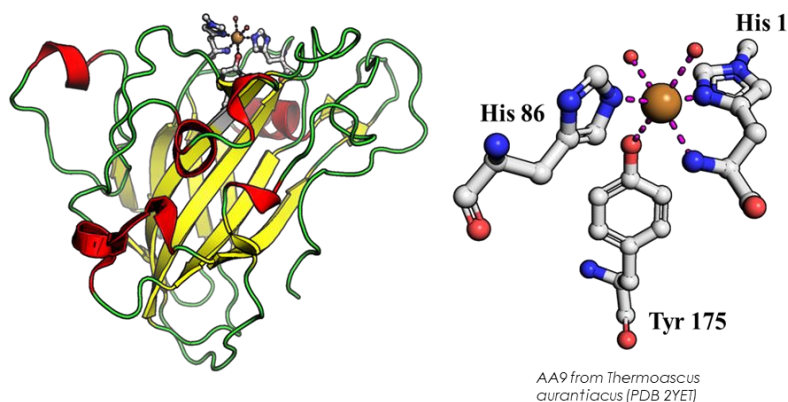


Fig. 6. Structure of family AA9 LPMO from *Thermoascus aurantiacus* (PDB 2YET) and its catalytic site, with key residues in ball-and-stick representation: carbon atoms in grey, nitrogen atoms in blue, and oxygen atoms in red. The copper atom is shown as a golden sphere and the coordinated water molecules are shown in red.

LPMOs typically oxidize and break glycosidic bonds in the presence of molecular oxygen and an external electron donor. It has been reported that both small and large molecules can function as electron donors for LPMOs. Small molecules include ascorbic acid and gallic acid, while large molecules include fungal cellobiose dehydrogenase (Phillips et al., 2011). Additionally, other enzymes, such as polyphenol oxidase and glucose-methanol-choline oxidoreductase, can act as electron donors for LPMOs (Frommhagen et al., 2017, Kracher et al., 2016). Lignin and lignin-derived substances may also serve as electron donors

(Dimarogona et al., 2012; Cannella et al., 2012; Westereng et al., 2015; Tang et al., 2022). The catalytic activity of LPMOs also depends on the availability of molecular oxygen, or hydrogen peroxide (Bissaro et al., 2017). The exact identity of the oxidants and reductants involved in LPMO catalysis in nature remains uncertain. Nevertheless, enzyme producers have enhanced their cellulolytic enzyme cocktails by incorporating LPMOs (Müller et al., 2015).

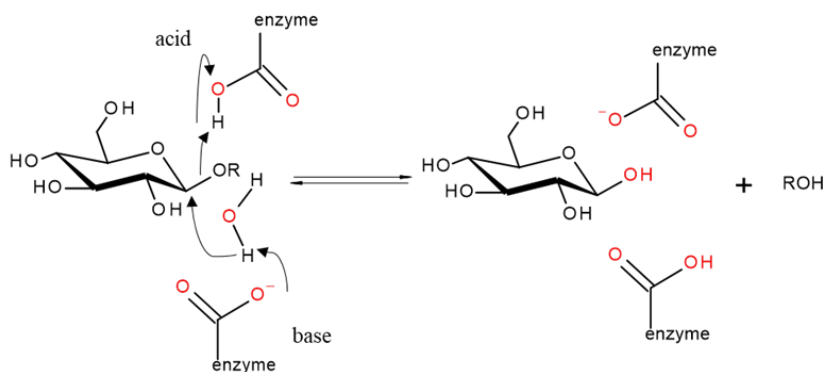


Fig. 7. The hydrolysis mechanism of glycoside hydrolases.

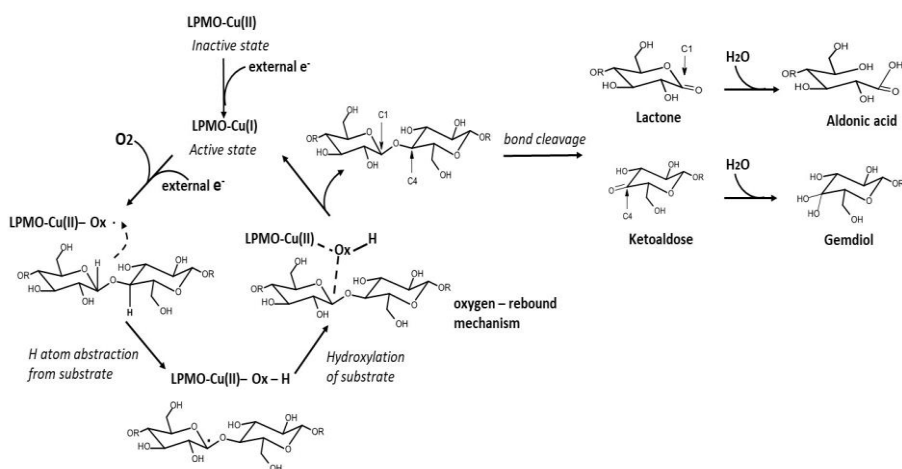


Fig. 8. Schematic mechanism of an LPMO-catalyzed reaction. “Ox” refers to copper(II)-superoxide (LPMO-Cu(II)-O-O-) or copper(II)-oxyl (LPMO-Cu(II)-O-) intermediates.

Microbial fermentation

In a narrow sense, fermentation refers to a metabolic process, taking place in the absence of oxygen, in which microorganisms convert organic compounds (e.g. carbohydrates) into simpler substances and release energy. Fermentation is used extensively in food and beverage production (e.g., bread, beer, wine, yogurt) (Leeuwendaal et al., 2022) and in industrial applications (e.g., biofuel production) (Voidarou et al. 2020). The initial stage of fermentation is glycolysis, in which glucose is broken down into pyruvate, producing small amounts of ATP and NADH in the process. In alcoholic fermentation, the pyruvate is then converted to acetaldehyde and CO₂, and acetaldehyde is reduced to ethanol in a reaction in which NAD⁺ is regenerated through oxidation of NADH (Voidaru et al., 2020). Hexose (six-carbon) and pentose (five-carbon) sugars, derived from enzymatic saccharification of biomass, can undergo fermentation by yeast, bacteria, and filamentous fungi to yield desired products, typically alcohols and/or carboxylic acids. Bioethanol and biobutanol are typically produced through the fermentation of carbohydrates using yeast and bacteria (Mohd Azhar et al., 2017).

Saccharomyces cerevisiae, commonly known as baker's yeast or brewer's yeast, is a species of yeast that has been widely used in baking and brewing for thousands of years, and it is widely utilized in industrial bioethanol production from glucose (Chandel et al., 2011). One notable characteristic of *S. cerevisiae* is its ability to convert sugars to ethanol under both anaerobic and aerobic conditions. Even when oxygen is present and respiration could occur, *S. cerevisiae* prefers alcoholic fermentation when glucose concentrations are high, a phenomenon known as the Crabtree effect (De Deken, 1966). Yeasts that exhibit this trait are known as Crabtree-positive yeasts. This metabolic feature is important in various industrial applications, particularly in processes where ethanol production is desired.

Glucose is the most favored sugar for microorganisms during fermentation because most industrial microorganisms, such as *S. cerevisiae*, have evolved to preferentially consume hexose sugars, like glucose, over pentose sugars (Oreb et al., 2012). Consequently, fermenting a mixture of different sugars, known as mixed sugar fermentation, presents more challenges for yeast. However, genetic engineering can enable yeast strains to ferment other sugars as well. For example, CelluX™₄ is a genetically modified strain of *S. cerevisiae* that has been engineered to ferment xylose, making it suitable for the production of cellulosic ethanol. This capability was also demonstrated in the studies presented in Papers III and IV.

In addition to *S. cerevisiae*, other microorganisms are of significant interest for industrial fermentation processes. For example, the bacterium *Clostridium acetobutylicum* is a Gram-positive, endospore-forming anaerobe that serves as a model organism for solventogenic clostridia. It is particularly well-known for its ability to perform acetone-butanol-ethanol (ABE) fermentation (Dürre, 1998; Lee et al., 2012) This fermentation pathway was developed and optimized by Chaim Weizmann during World War I, making *C. acetobutylicum* a key organism in the production of solvents. *Zymomonas mobilis* is a Gram-negative bacterium that is sometimes used for alcoholic fermentation as an alternative to *S. cerevisiae*. As *S. cerevisiae*, *Z. mobilis* has been engineered for fermentation of pentose sugars from biomass (Zhang et al., 1995; Xia et al., 2019).

Process configurations

The production of biofuel or other bio-based products can be accomplished through several process strategies which are illustrated in *Fig. 9*. There used to be two major alternative strategies that were used

to perform enzymatic saccharification and fermentation of lignocellulosic biomass: (1) separate hydrolysis and fermentation (SHF), in which enzymatic hydrolysis of pretreated biomass and microbial fermentation are carried out sequentially as separate steps, and (2) simultaneous saccharification and fermentation (SSF), in which the enzymatic and microbial processes are carried out in the same step (Tomás-Pejó et al., 2008; Wingren et al., 2003). The advantages of SHF are that hydrolysis and fermentation are operated in a way that is optimal for each process, especially with respect to the temperature. Enzymatic saccharification is typically carried out at 45 to 50 °C while microbial fermentation with yeast is typically carried out at 30 to 35 °C. In contrast, in SSF the temperature is usually kept at the highest temperature the microorganism can tolerate, typically no more than 35 °C for yeast, implying that the enzymatic saccharification is carried out at suboptimal conditions. However, in SHF the enzymatic saccharification will be affected negatively by end-product inhibition of cellulolytic enzymes by sugar. This is not likely to occur in SSF because of the simultaneous consumption of sugar by the fermenting microorganism. Using conventional cellulolytic enzyme preparations, higher yields of ethanol were typically found when pursuing the SSF strategy (Olofsson et al., 2008; Tomás-Pejó et al., 2008; Erdei et al., 2010).

A tentative advantage with SHF is that it makes it possible to separate the liquid and the solid phases after the enzymatic saccharification and prior to fermentation. That would facilitate recirculation of the yeast, as it would be easier to collect the yeast without also collecting the solid hydrolysis residue.

Another process approach is called Consolidated Bioprocessing (CBP) and was introduced by Lynd et al. (2002). The concept of CBP is to develop or utilize microorganisms capable of both degrading cellulose into fermentable sugars and then fermenting the sugars into desired

products within a single bioreactor system. The aim is to simplify the process and reduce costs associated with biomass conversion (Lynd et al., 2005; Olson et al., 2012). The largest difficulty in implementing CBP lies in developing a single microorganism that is capable of efficiently performing all necessary steps. Thus, there is still a need for further development (Singhania et al., 2022).

LPMO has the potential to improve the efficiency of bioconversion of lignocellulosic biomass. Through its ability to work synergistically with conventional cellulases and enzymes with auxiliary activity, such as hemicellulases, LPMO could boost the breakdown of cellulose, resulting in more efficient conversion of biomass into monosaccharide sugars. Therefore, LPMO is nowadays a common component in commercial enzyme preparations designed for bioconversion of cellulosic substrates. However, LPMO requires a chemical environment with both a suitable oxidant and a suitable reductant. Thus, the process strategy used for biochemical conversion needs to be reexamined because of the inclusion of LPMO (Cannella and Jorgensen, 2014). One reason is because an adequate supply of reductant has to be present, and another reason is the oxidant. Even if *S. cerevisiae* is a Crabtree-positive yeast, there are typically no measures taken to provide any supply of oxygen to the fermentation reaction. There are also other microorganisms than yeast that are used, which are not Crabtree positive, and microorganisms also differ with respect to their resistance to phenolic lignin-degradation products (that could potentially serve as reductants for LPMO) and with respect to their tolerance to the presence of oxygen in reaction mixtures. A process called Hybrid Hydrolysis and Fermentation (HHF) could be a promising alternative, as explored in Paper III. In this process configuration, an initial saccharification step is performed at high temperature (50-55 °C) and potentially with continuous aeration to enhance the activity of LPMOs. This is followed by a fermentation step

at a lower temperature, typically 30-35 °C, which is suitable for yeast fermentation. In the study presented in Paper III, different aeration periods (24 h and 48 h) were compared to evaluate their impact on the process. Fig. 9 illustrates the various process configurations discussed in this study.

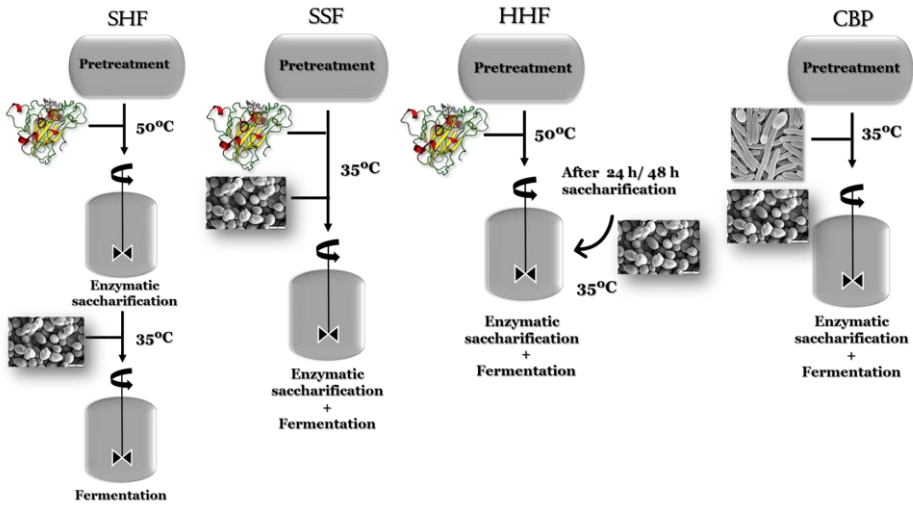


Fig. 9. Different process configurations in the biochemical conversion of lignocellulosic biomass.

Aims of study

The doctoral project has been focused on the redox conditions that are used in the processing of lignocellulosic biomass through biochemical conversion, and particularly the conditions used for LPMO-supported saccharification reactions. The study material has been softwood pretreated using steam explosion with an acid catalyst, as softwood is the most prevalent type of lignocellulosic biomass in Sweden (>80% of the standing volume of Swedish forests) (Forest Statistics, 2023). The pretreatment and some other experiments have been performed using the Biorefinery Demo Plant in Örnsköldsvik in northern Sweden. The pretreatment methodology has not been specifically designed for the project, and often small portions of larger pretreatment campaigns have been set aside to carry out the experiments described in the papers.

The specific objectives were:

I: Study lignin and water-soluble lignin-degradation products as reductants in LPMO-supported enzymatic saccharification to investigate their ability to promote LPMO-supported catalysis.

II: Explore how the fraction of solids, specified as water-insoluble solids (WIS), influences LPMO-supported enzymatic saccharification. The fraction of WIS was varied in the range 12.5% (w/w) to 17.5% (w/w).

III: Evaluate different process configurations, including SSF and HHF (a pre-hydrolysis stage before fermentation) in conjunction with LPMO-driven saccharification at a demonstration plant. *S. cerevisiae* yeast was employed for fermentation.

IV: Understanding potential effects of aeration on inhibitors using *S. cerevisiae* yeast to assay the fermentability.

Materials and methods

Raw materials and pretreatment

As raw materials were pretreated in the Biorefinery Demo plant, the focus was mainly on separation of fractions and conditioning of slurries and hydrolysates for subsequent studies. For instance, centrifugation, grinding, sieving, and pH adjustments were needed in order to prepare the relevant starting material for each experiment. As described in Papers I and IV, centrifugation was commonly employed to achieve solid-liquid separation. The separated solid fractions were washed thoroughly to remove residual water-soluble substances from the pretreatment. Grinding and sieving were conducted after air-drying of materials to get samples with particle sizes in the same interval. With respect to the liquid phase, filtration was performed to remove residual particles, thereby clarifying the liquid. This multi-step approach ensured that both solid and liquid fractions were suitable to meet the specific needs of the experiments.

Total solids (TS), soluble solids (SS), and water-insoluble solids (WIS) are terms that are commonly used to describe the fraction of solids in pretreated biomass. TS consists of both dissolved solids and suspended water-insoluble solids. The TS content is measured by drying the sample at high temperature until all water is removed. SS refers to the fraction of total solids that is dissolved in water. The SS content is usually measured by filtering the sample to remove all suspended water-insoluble solids and then evaporating the water from the filtrate to determine the weight of the dissolved solids. WIS, as discussed in Paper II, refers to the portion of total solids that does not dissolve in water, measured by first separating the water-insoluble portion of the sample through filtration or centrifugation, followed by drying the residues and weighing it to determine the amount of WIS present.

In-house laboratory reactor system

To perform the LPMO-supported enzymatic saccharification, an in-house laboratory reactor system described in Papers I and II was developed. The saccharification reactions were carried out in 125 mL gas wash bottles, into which air or nitrogen was introduced to establish aerobic or anaerobic conditions. Each gas line was divided into three strands using a three-way air distributor, followed by flow meters to ensure consistent flow rates across all strands. This set-up made it possible to run two sets of triplicates in parallel (one with air and the other with N_2). To avoid foaming, the flow rate of the gas addition was adjusted according to what was practically feasible. In this set-up, the gas flow rate for the enzymatic saccharification was performed at one vvm (volume per reaction mixture volume per min). The six reaction bottles were placed on a multipoint stirrer that was immersed in a water bath to regulate the temperature and to provide proper mixing during the saccharification. An illustration of the reaction system is shown in Fig. 10.

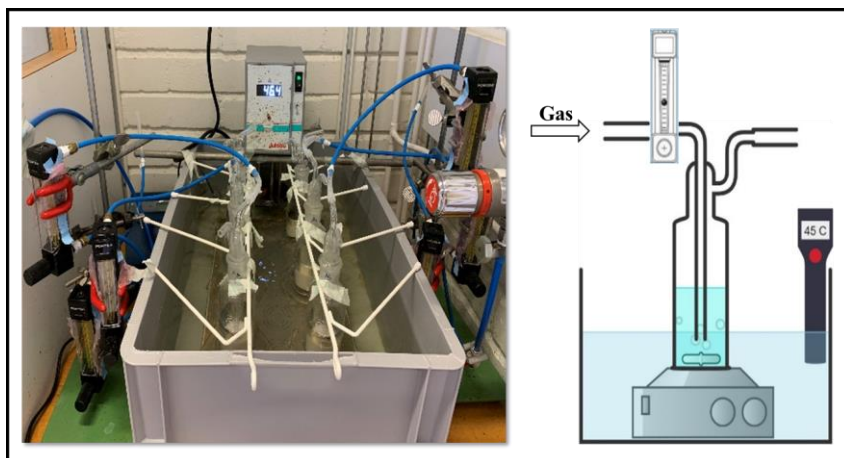


Fig. 10. In-house laboratory reactor system used for studies of enzymatic saccharification. The system consisted of six bottles, of which three were fed with air and three were fed with N_2 .

To perform the fermentation series experiment with gas addition described in Paper IV, the in-house laboratory system was modified for performing the continuous gas addition before conducting the fermentation in the same incubator later on. As Fig. 11 shows, in that study, there were six parallel 55 mL serum bottles, each containing 30 mL reaction mixture, sealed with rubber caps. The bottles were partially immersed in the water bath. The gas was supplied through sterile needles, while a shorter needle was inserted into the rubber cap to release carbon dioxide during fermentation. A flow rate of 0.2 vvm was selected based on previous studies (Paper II) and based on practical issues related to the smaller reaction bottles used for this study. The temperature control and mixing using magnetic stirrer bars was the same as described previously.



Fig. 11. A modified laboratory reactor for fermentation experiments.

Different process configurations in demonstration scale

Simultaneous saccharification and fermentation (SSF) with older generations of cellulase preparations has been shown to result in high yields of ethanol (Erdi et al., 2010, Tomás-Pejó et al., 2008). However, inclusion of LPMO in enzyme preparations could affect the choice of process configuration (Cannella and Jorgensen, 2014). Thus, experiments were performed using an alternative process configuration, hybrid hydrolysis and fermentation (HHF), in a comparison with SSF (Paper III).

In HHF, the reaction is initiated with enzymatic saccharification performed at high temperature, which is then decreased (typically to 30°C–35°C) before the inoculation with the fermenting microorganism, typically yeast. After inoculation of the microorganism, the enzymes can continue working, although the reaction conditions are no longer optimal for the enzymatic part of the process.

In our experiments, the HHF also included aeration in the initial phase before addition of the yeast. Two different time periods (24 h and 48 h) for the initial phase were compared. The experiments were conducted in demonstration scale using 10 m³ bioreactors at the Biorefinery Demo Plant in Örnsköldsvik in northern Sweden. Besides addressing the trade-off between, on the one hand, boosting the LPMO activity by using higher temperature and gas addition in the initial stage of the HHF, and, on the other hand, minimizing end-product inhibition of cellulolytic enzymes by using the SSF approach, the investigation also addressed potential effects on fermentation inhibitors and inhibition of *S. cerevisiae* yeast. The yeast used in the experiments was a commercially available engineered xylose-fermenting strain of *S. cerevisiae*, the CelluX™4.

Analysis of the solid phase of pretreated biomass

Compositional analysis based on sulfuric acid

The composition of lignocellulosic biomass, specifically the carbohydrate content (typically arabinan, galactan, glucan, mannan, and xylan) and the lignin content, can be determined using a two-step treatment with sulfuric acid. The approach has been used in wood chemistry for a long time, but commonly used protocols today include the TAPPI standard T249 and the National Renewable Energy Laboratory (NREL) report NREL/TP-510-42618. Before performing two-step acid treatment, the extractives of the biomass are removed by using extraction with organic solvent. The traditional approach is using Soxhlet extraction. Compared to Soxhlet extraction, accelerated solvent extraction (ASE) can be a better option, as using a pressurized system at high temperature and several cycles with fresh solvent efficiently removes the extractives. The choice of extraction solvent can be varied depending on the biomass and the temperature used. In some cases, a single solvent, such as acetone or ethanol, is used for extraction. However, using a mixture of solvents with varying polarity can be a better way to target both lipophilic and hydrophilic substances. For softwood, a non-polar solvent is often included, as using only a polar solvent, such as ethanol, may give inadequate extraction results. After extraction, the biomass is treated with 72% sulfuric acid at 30 °C for 1 h. The acid concentration is then reduced to 4% through dilution with water, and the suspension is subjected to autoclaving at 121 °C for 1 h (Sluiter et al., 2012). This process hydrolyzes cellulose and hemicellulose into their monosaccharides, including arabinose, galactose, glucose, mannose, and xylose. According to the NREL protocol (Sluiter et al., 2012), the quantification of the monosaccharides is conducted using high-performance liquid chromatography (HPLC) with refractive index detector (RID). However, high-performance anion-exchange

chromatography (HPAEC) with pulsed amperometric detection (PAD) offers several advantages for quantification of the monosaccharides, providing superior separation and resolution, particularly with respect to distinguishing between xylose and mannose. Also, the sensitivity in HPAEC-PAD is superior compared to the conventional HPLC-RID method.

After hydrolysis using sulfuric acid and hydrolysis and solubilization of carbohydrates, the solid phase predominantly consists of lignin, which is not affected by acid as easily as carbohydrates. However, the liquid phase will contain some lignin-degradation products that were solubilized during the treatment with sulfuric acid. The fraction of the lignin that was solubilized is referred to as acid-soluble lignin or, for short, ASL. The ASL is quantified using UV spectrophotometry. The benzene ring in lignin-degradation products, including phenolic compounds, absorbs UV light strongly due to its conjugated π -electron system, and quantification is typically made at around 240 nm.

The acid-insoluble lignin left in the solid phase is also known as Klason lignin, after Peter Klason, who was one of the pioneers in wood chemistry in the early 20th century (Marton and Adler, 1966). The Klason lignin is determined gravimetrically by measuring the solid residue remaining after the acid treatment. To ensure accurate measurements, the moisture in the solid residue must be removed by drying in an oven at 150 °C overnight. Following the drying, the sample should be handled with care and stored in a desiccator to prevent moisture uptake, which could otherwise alter the weight of the residues.

Inorganic substance is determined by ashing at 575 °C for 24 h. Some forms of biomass contain large fractions of inorganic material, but as pure wood typically contains very little inorganic material, determination of inorganic substance is sometimes ignored in the

analysis of pure wood. However, bark contains higher levels of inorganic substance than wood.

Pyrolysis-gas chromatography/mass spectrometry

Lignin and carbohydrates in lignocellulosic biomass can be analyzed using pyrolysis gas chromatography combined with mass spectrometry (Py-GC/MS) (Meier et al., 1992). During pyrolysis, the biomass is decomposed into smaller fragments, which provide structural information about the original material. The fragments are then separated using gas chromatography, while mass spectrometry is used for identification and quantification of specific components (Kusch, 2012).

Apart from providing information about the carbohydrate:lignin ratio, Py-GC/MS also provides information about the composition of the lignin, i.e., the ratio of syringyl, guaiacyl, and *p*-hydroxyphenyl units. This technique is also useful for estimation of pseudo-lignin, which is a Klason-lignin positive by-product formed from carbohydrates (Yao et al., 2022). In compositional analysis using sulfuric acid, pseudo-lignin will be misinterpreted as Klason lignin. The presence of pseudo-lignin can be estimated by comparing the results from Py-GC/MS with those obtained from compositional analysis using sulfuric acid. However, the comparison is not straightforward, as the results from Py-GC/MS analysis are typically peak area fractions rather than mass fractions.

Fourier-transform infrared spectroscopy

Fourier-transform infrared spectroscopy (FTIR) is used to identify chemical substances or their functional groups by measuring their interaction with infrared radiation, through absorption, emission, or reflection. The generated infrared absorption spectrum is unique for each molecule, identifying the characteristic chemical bonds. The

spectrum serves as a distinctive fingerprint and provides information about different components in the sample. FTIR is a simple and fast analytical method that offers a qualitative and semi-quantitative analysis of lignocellulosic biomass (Tucker et al., 2001; Liebmann et al., 2010).

The wavenumber interval 800 to 1800 cm^{-1} is typically used to identify functional groups in cellulose, hemicelluloses, and lignin. Lignin exhibits characteristic absorption bands at approximately 1510 cm^{-1} and 1600 cm^{-1} , corresponding to aromatic carbon-carbon bonds (Sarkanen and Ludwig, 1971). The band at 1510 cm^{-1} is primarily associated with guaiacyl units, while the band at 1595 cm^{-1} is linked to syringyl units (Faix, 1991; Faix and Böttcher, 1992). Although FTIR provides limited carbohydrate information, it can offer insight into cellulose crystallinity. The intensity ratio of the bands at 1429 cm^{-1} and 897 cm^{-1} , known as the Total Crystallinity Index (TCI), distinguishes between crystalline and amorphous regions of cellulose. The band at 1429 cm^{-1} is associated with crystalline regions, whereas the band at 897 cm^{-1} is linked to amorphous regions (Nelson and O'Connor, 1964).

X-ray diffraction

X-ray diffraction (XRD) is primarily used to analyze the atomic and molecular structure. It provides detailed information about the arrangement of atoms or molecules within crystalline regions, allowing for the determination of crystal structure. XRD provides more comprehensive insights into the crystallinity of cellulose structures (Park et al., 2010) compared to other techniques, for example, solid-state ^{13}C -NMR spectroscopy and FTIR spectroscopy. The crystallinity index (CrI) is calculated from the ratio of the height of the 002 peak (I_{002}) and the height of the minimum (I_{AM}) between the peaks 002 and 101 (Park et al., 2010).

In the investigations presented here, the data collection was continued until the diffractograms were smooth. The collected diffractograms underwent background removal treatment. Key points, I_{AM} and I_{002} , were identified with the aid of a 4th degree polynomial fit, which is necessary for calculating the crystallinity index (CrI). The CrI was determined by the intensity ratio between I_{AM} and I_{002} , expressed in counts per second using Eq. 1.

$$\text{CrI (\%)} = \frac{(I_{002}-I_{AM})}{I_{002}} \times 100 \quad (\text{Eq. 1})$$

In Eq. 1, " I_{AM} " represents the intensity of diffraction of non-crystalline material, which is defined as the minimum of the valley between the main cellulose peaks in the range 17.8°-19.8°. " I_{002} " refers to the maximum intensity of the peak that corresponds to the plane with the Miller indices 002 at a 2θ angle of 22°-24°.

NMR spectroscopy

2D-HSQC NMR (two-dimensional heteronuclear-single-quantum-coherence nuclear magnetic resonance) spectroscopy can provide detailed insights into the structural composition of lignocellulosic biomass. This method allows for the identification and differentiation of various cellulose structures by correlating proton (^1H) and carbon (^{13}C) signals. It is especially useful for analyzing lignin, enabling the differentiation of various lignin subunits and interunit linkages, such as β -O-4, within the lignin polymer (Lu and Ralph, 2011). Since the samples used for analysis with 2D-HSQC NMR are typically in liquid form, the biomass needs to be dissolved, for instance, using deuterated solvents (DMSO-d₆, i.e., deuterated dimethyl sulfoxide, or acetone-d₆). Lignin from pretreated biomass may exhibit very poor solubility, which is a challenge when doing this type of analysis.

Solid-state studies of cellulosic materials are often performed using ^{13}C cross-polarization/magic-angle-spinning (CP/MAS) NMR spectroscopy (Foston et al., 2011). As solid-state NMR does not require solubilization of the sample, it is ideal for studying materials with poor solubility. Compared to direct-polarization NMR, cross-polarization NMR reduces the time required for the analysis with more than a factor of 50 (Johnson and Schmidt-Rohr, 2014). The "magic angle" refers to the spinning of the sample around an axis with a tilt of 54.7° in relation to the magnetic field in order to obtain spectra with high resolution (Reif et al., 2021).

Additionally, this technique can differentiate between crystalline and amorphous regions within a sample. In crystalline regions, the cellulose chains are well-ordered, exhibiting a high degree of symmetry, leading to a narrower peak at 89 nm. Amorphous regions exhibit a broader peak at 84 nm because of the less ordered structure. The crystallinity index was calculated according to Eq. 2.

$$\text{CrI (\%)} = \frac{\text{Int. (89)}}{\text{Int. (89)} + \text{Int. (84)}} \times 100 \quad (\text{Eq. 2})$$

Scanning electron microscopy

Scanning electron microscopy (SEM) is a useful technique for capturing high-resolution, three-dimensional images of solid biomass (Joy et al., 1992). Compared to optical microscopes that rely on light, SEM utilizes a focused beam with high-energy electrons to interact with the surface of the sample, to provide detailed images that reveal the microstructure of the solid biomass, offering a deeper understanding of the surface characteristics of material. The resolution of SEM is in the range of 1 to 10 nm, but it varies depending on the type of SEM that is used. For example, field emission SEM (FE-SEM) has higher resolution than conventional SEM.

Brunauer-Emmett-Teller (BET) analysis

BET analysis can provide insights into the surface properties of the biomass, focusing on surface porosity and the specific surface area. The technique is named after its developers, Stephen Brunauer, Paul Emmett, and Edward Teller, who introduced it in 1938 (Brunauer et al., 1938). The BET method is based on the adsorption of an inert gas (typically nitrogen), onto the surface of the material at a constant temperature (typically around the temperature of liquid nitrogen -196 °C). The amount of the gas adsorbed can be measured as a function of the relative pressure of the gas. To ensure the accuracy of the measurements, the samples need to undergo a degassing procedure to remove any contaminants that might have adsorbed onto the surface.

Simons' staining analysis

Simons' staining analysis is used to evaluate the accessible surface area for enzymes in the degradation of cellulosic biomass. There are two dyes involved: Direct Orange (DO, Pontamine Fast Sky Orange 6RN) and Direct Blue (DB, Pontamine Fast Sky Blue 6BX). The measurement depends on the differential adsorption of these dyes based on their molecular size. The larger DO dye (> 100 kDa) is of a similar size as many cellulases and has also been found to have higher affinity to cellulose than to hemicellulose and lignin (Chandra and Saddler, 2012). The size of the DB dye (~ 993 Da) is much smaller than that of an enzyme.

The dye concentrations in the supernatant were determined using Lambert-Beer's law, as described by the equations provided Eqs. 3 and 4 (Esteghlalian et al., 2001):

$$A_{455\text{nm}} = \epsilon_{\text{DO}/455} \times L \times C_{\text{O}} + \epsilon_{\text{DB}/455} \times L \times C_{\text{B}} \quad (\text{Eq. 3})$$

$$A_{624\text{nm}} = \epsilon_{\text{DO}/624} \times L \times C_{\text{O}} + \epsilon_{\text{DB}/624} \times L \times C_{\text{B}} \quad (\text{Eq. 4})$$

In Eqs. 3 and 4, "A" refers to the absorption at 455 and 624 nm, respectively. Here, the extinction coefficients (Direct Orange, ϵ_{DO} ; Direct Blue, ϵ_{DB}) of the dyes at the respective wavelengths are ($L\ g^{-1}\ cm^{-1}$): $\epsilon_{DO/455}$, 35.62; $\epsilon_{DB/455}$, 2.59; $\epsilon_{DO/624}$, 0.19; $\epsilon_{DB/624}$, 15.62. L is the path length (cm) of the microplates used. C_O and C_B are the concentration of the orange dye and the blue dye, respectively.

Analysis of the liquid phase of pretreated biomass

Liquid chromatography

Monosaccharides can be analyzed using both high-performance liquid chromatography (HPLC) and high-performance anion-exchange chromatography (HPAEC). Furthermore, substances such as ethanol, glycerol, and acetate are often analyzed using HPLC.

HPLC with Bio-Rad Aminex HPX-87H and HPX-87P columns and refractive index detector (RID) is typically used for quantification of monosaccharides (primarily Aminex HPX-87P), organic acids, and alcohols (primarily Aminex HPX-87H). The separation mechanism using resin columns is complex and involves ion exclusion, size exclusion, and ligand exchange. The separation of the different compounds is based on their chemical properties which govern the interactions with the resin.

Monosaccharides are neutral molecules and, therefore, are not ionized in an acidic mobile phase. They will not be retained on resin columns through ionic interactions but are rather separated based on their exclusion from the resin pores. Thus, the separation is mainly based on their molecular size, as smaller molecules can be retained by the resin for a longer time, while larger molecules elute faster (Alt et al., 2024). However, ethanol is separated based on different mechanisms. Here, the separation is mainly based on its hydrophilic properties and its exclusion from the ion-exchange sites of the resin. There will be a minor

contribution from hydrophobic interactions between the ethanol molecules and the resin, which can slightly affect the retention time. Ethanol is less polar than monosaccharides, leading to a different interaction with the column. Aliphatic acids, for instance, formic acid, acetic acid, and levulinic acid, ionize to different extent depending on the pH of the mobile phase. The separation of aliphatic acids by resin columns is mainly based on ion exclusion. The stationary phase of the column interacts with the acids based on their ionic state. A more ionized acid interacts more strongly with the stationary phase, leading to different elution time for different acids.

Furan aldehydes, such as furfural and HMF, can be quantified using an HPLC with a C18 column and a UV detector. A C18 column is a reverse-phase column packed with silica particles bonded with octadecylsilane (C18) groups. The stationary phase is nonpolar (hydrophobic C18 chains), while the mobile phase is polar. Furfural and HMF have relatively low polarity due to their furan rings, which account for much of the interactions with the C18 stationary phase. By adjusting the polarity of the mobile phase, the analytes are eluted with different retention times. HPLC is generally robust and cost-effective and suitable for standard laboratory settings. However, RID is not a sensitive detection method and low concentrations of monosaccharides are difficult to analyze. Separation of very similar analytes, such as monosaccharides, is also challenging. For instance, xylose and mannose may not be well separated, since they have similar hydrophilic properties, potentially leading to an overlapping peak.

HPAEC-PAD is typically a better option than HPLC-RID, as it provides higher sensitivity and resolution in analysis of complex mixtures of monosaccharides, for example superior separation of xylose and mannose. It separates monosaccharides according to their charge properties using a strong anion-exchange column. At high pH levels,

monosaccharides with pKa values in the range of 12-14 exist in their anionic forms, allowing to perform the separation on anion-exchange columns. Pulsed amperometric detection (PAD) provides high sensitivity. It is also preferable for analyzing acidic sugars, for instance, gluconic acid, which is a C1 oxidation product in LPMO-supported enzymatic saccharification. Thus, the method offers an indication of LPMO activity in reaction mixtures. Additionally, HPAEC may be coupled with a conductivity detector for analyzing aliphatic acids. The conductivity detector measures the electrical conductivity of the eluent as it passes through the detector and the conductivity is influenced by the ionic concentration in the solution. As aliphatic acids contribute the ionic strength of the solution, the detector measures the changes in the conductivity due to the presence of these anions.

Mass spectrometry

Mass spectrometry is a powerful analytical tool used for identifying and quantifying chemical substances, which offers detailed information on molecular mass and structure (El-Aneed et al., 2009). Briefly, the analyte is ionized using, for instance, electron ionization, electron spray ionization (ESI), or matrix-assistant laser desorption/ionization. Then, the ionized species are separated based on their mass-to-charge ratio (m/z) in the mass analyzer, for instance, quadrupole, time-of-flight, and ion trap. The separated ions give rise to mass spectra. Tandem mass spectrometry (MS/MS) involves multiple stages of mass analysis. In MS/MS, the selected ions in the first stage (MS₁) are further fragmented at a collision cell to get smaller fragments in the second stage (MS₂). MS/MS offers a more in-depth analysis by providing fragment patterns, quantifying analyte with higher accuracy.

The quantification of inhibitory compounds, for instance formaldehyde, acetaldehyde, coniferyl aldehyde, vanillin, and

benzoquinone, was performed using ultra-high-performance liquid chromatography coupled with electrospray ionization triple quadrupole mass spectrometry (UHPLC-ESI-QqQ-MS). In order to improve the sensitivity and selectivity of the analysis, derivatization using 2,4-dinitrophenylhydrazine (DNPH) was done. That is because many carbonyl-containing compounds (aldehydes or ketones) have poor ionization efficiency in mass spectrometry, or, with low volatility, they decompose at higher temperatures. The derivatization reaction with DNPH is specific for carbonyl groups, involving a nucleophilic addition of the hydrazine group of DNPH to the carbonyl group of aldehydes or ketones, followed by a condensation reaction to form a yellow- or orange-colored hydrazone. The resulting hydrazone derivatives are generally more stable than the substances from which they are derived. Due to the reaction only works with aldehydes and ketones, its applicability is limited to substances that contain these functional groups.

While DNPH is quite popular for LC analysis, trimethylsilyl reagent is used to increase the volatility of polar and non-volatile compounds, making them more amenable for GC analysis. This is especially useful for carbohydrates, fatty acids, and amino acids (Zarate et al., 2016).

Total phenolic content (TPC)

Measurement of total phenolic content (TPC) is typically based on the ability of phenolic compounds to reduce certain reagents, leading to a color change that can be quantified using spectrophotometry. Folin-Ciocalteu's reagent (FC) (Singleton et al., 1999) is a common reagent for performing the analysis. This assay relies on the reduction of Folin-Ciocalteu reagent by phenolic compounds under alkaline conditions, resulting in a blue-colored molybdenum-tungsten complex that absorbs at a specific wavelength, usually around 760-765 nm. The method is a widely used and effective technique for estimating the total phenolic content of the samples despite its limitations in specificity. With regard to lignocellulosic hydrolysates, the Folin-Ciocalteu assay was found to have certain advantages in a comparison with other methods (Persson et al., 2002). Vanillin is typically used as standard, as vanillin is typically one of the most common phenolics in lignocellulosic hydrolysates.

Total aromatic content (TAC)

A general overview of groups of by-products can also be obtained by determination of the total aromatic content (TAC). TAC is a spectrophotometric method used to estimate the total aromatic (such as phenolics) and heteroaromatic (such as furans) compounds in hydrolysates based on UV absorption at 280 nm.

Total carboxylic acid content (TCAC)

TCAC is a titration method that mainly measures substances that have carboxylic acid functional groups (-COOH). Many of these substances have a pKa at around 4.5 and by starting the titration at acidic pH and then increase the pH to neutral, using a solution of sodium hydroxide, the carboxylic acid content can be estimated (Wang et al., 2018a).

Results and discussion

The role of lignin as reductant (Paper I)

LPMOs catalyze the oxidative cleavage of glycosidic bonds in cellulose, requiring molecular oxygen as a co-substrate and an electron donor (Hemsworth et al., 2015). Lignin has been suggested as a potential electron donor for LPMO and would be preferred in an industrial context, both because of practical reasons (it is already in the reaction mixture) and economic considerations (no need to use costly additives, such as ascorbic acid) (Cannella et al., 2012; Westereng et al., 2015). During enzymatic saccharification, it is a possibility to either include or exclude the liquid phase of the pretreated biomass. It is common to use the slurry, which is the mixture of the solid and liquid phases obtained after the pretreatment, as substrate in the enzymatic saccharification. In this scenario, both water-insoluble lignin in the solid phase and water-soluble lignin in the liquid phase are present and can potentially act as reductants in LPMO-supported saccharification. However, hemicellulose hydrolysate (also referred to as pretreatment liquid) can be separated from the solids and used for other purposes than the solids. Potential advantages with separation of the liquid and solid phases prior to enzymatic saccharification of the cellulose include that (i) the pentose-rich hemicellulose hydrolysate can be used for pentose conversion whereas hexose sugar from the cellulose in the solid fraction (i.e., glucose) can be used in a dedicated hexose conversion process based on a clean sugar stream, (ii) water-soluble pretreatment by-products that inhibit enzymes and microorganisms will be removed prior to enzymatic saccharification and hexose fermentation, and (iii) enzymatic saccharification of cellulose can be initiated without the presence of saccharides causing end-product inhibition of cellulases.

The study presented in Paper I was focused on how the electron donor capacity was divided between water-insoluble lignin and water-soluble lignin-degradation products using industrially relevant lignocellulosic biomass (steam-exploded softwood). In addition, we investigated the effects of continuous gas addition using a novel laboratory system based on six parallel reactions with controlled gas addition rate. In other studies (Müller et al., 2015; Chylenski et al., 2017), the gas phase was not controlled and the molecular oxygen required for the LPMO reaction was derived from the headspace of the reactor. Reactions without the pretreated solids (PS) present were carried out using pretreatment liquid (PL) and with Avicel as substrate. Avicel is a preparation of microcrystalline cellulose that is often used as model substrate in studies of cellulases.

The results clearly showed that higher glucose yields were always obtained using aerobic conditions (gas flow of air), both for saccharification of Avicel suspended in PL and for saccharification of PS suspended in a buffered aqueous solution (Table 3 in Paper I). The results also clearly showed that both the PS and the PL served as reductants in LPMO-supported saccharification of cellulose. LPMO oxidation products related to C1 and C4 were further indicators of LPMO activity. For both sets of reactions performed under aerobic conditions, both oxidation products showed an increasing trend compared to the corresponding anaerobic conditions (Fig. 3 in Paper I). That the pH slightly decreased (Table 2 in Paper I) might possibly be due to formation of LPMO oxidation products (sugar acids). This is also a possible explanation for the higher TCAC (total carboxylic acid content) values.

The chemical analysis of the solid and liquid phases was conducted to provide a better understanding of the PL and the PS, and the reactions that were taking place. The total lignin content of the PS was higher than

the glucan content (Table 1 in Paper I), which indicates partial degradation of hemicellulose to form pseudo-lignin, which is typically formed during pretreatment of biomass under acidic conditions (Yao et al., 2022). Also, there was little or no hemicellulose left in the solid phase. This indicates that the pretreatment conditions were relatively harsh, and that the solid phase primarily consisted of Klason lignin, cellulose, and pseudo-lignin. Reactions with Avicel and PS contained the same amount of solids. The glucan content was therefore different, as Avicel consists almost entirely of glucan, while the PS had a glucan content of 45%. Avicel would therefore be a much better substrate for enzymatic saccharification than PS, also because the cellulose in Avicel was not shielded from the action of cellulases by lignin and pseudo-lignin, as the cellulose in the PS. However, the reaction with PL also contained inhibitory by-products from the pretreatment (such as phenolics) and sugar causing end-product inhibition of cellulases. There was around 5 g/L phenolics in the PL, which would likely be inhibitory to enzymatic saccharification reactions based on results from previous studies (Wang et al., 2018a). Also, a study by Martin et al. (2018) showed much lower concentrations of phenolics (0.7 g/L – 1.6 g/L). Although the phenolic by-products probably had a negative effect on the saccharification reactions by inhibiting cellulases, they also had a positive effect in the presence of air by supporting LPMO-catalyzed reactions.

Saccharification at high solids loadings (Paper II)

High capital and operational expenditures (Capex and Opex) contribute to making bio-based products more expensive. Enzymatic saccharification at high solids loadings (i.e., with a dry-matter content > 15% w/w) has potential to improve the economic feasibility of biochemical conversion process. Higher sugar concentrations would result in higher product titers and lower energy demand for down-

stream processing, for instance distillation of ethanol. However, it has been observed that glucose yields show a decreasing trend with increasing solid loadings. As 15 % w/w is the criterium for high solids loadings, we performed one set of reactions with lower solids loadings (12.5% WIS), and another set of reactions with higher solids loadings (17.5% WIS). As mentioned in the Materials-and-Methods section, WIS refers to water-insoluble solids, which are not dissolved in water. As in the study presented in Paper I, the catalytic action of LPMO was sustained with an oxidant (molecular oxygen from air) and lignin-based reductants (lignin in the solid phase and lignin-degradation products in the liquid phase). A comparison with continuous gas addition, with air and N₂, was included to give a better understanding of the potential contribution of LPMO to the saccharification. The aim of the investigation was to obtain a better understanding of whether the positive effect of LPMO would be large enough to compensate for the negative effect caused by high solids loadings.

Enzymatic saccharification reactions with varied WIS and with air always resulted in higher glucose production than corresponding reactions with N₂ (Table 2 in Paper II). Even though the glucose production after 72 h in aerated reactions increased with increasing WIS (Table 2 in Paper II), the glucan conversion showed a decreasing trend with increasing WIS (Fig. 1A in Paper II). It is, however, noteworthy that the positive effects of continuous gas addition with LPMO-supported saccharification were much larger than the negative effects of increased WIS. For example, the glucan conversion at high solids loading (17.5% WIS) under aerobic conditions (42%) was 50% higher than with low solids loading (12.5%) and anaerobic conditions (28%).

The effects of varied enzyme loadings (from 6% to 9%) on LPMO-supported saccharification were also evaluated. At lower levels (6-9%), the glucan conversion increased significantly for both air and N₂ when

the enzyme loading was increased (Table 2 and Fig. 1B in Paper II). However, when using even higher enzyme loadings the positive effects were still apparent for aerated reactions, but not for reactions with N₂. Similarly, the glucan conversion increased greatly for both air and N₂ when the enzyme preparation loading was increased from 6% to 9% (Fig. 1B in Paper II). However, when the enzyme preparation loading was increased from 9% to 12%, there was not much difference between the sets of reactions with N₂, both showing a glucan conversion of around 39%. In contrast, glucan conversion in the sets of reactions with air increased from 65% to 72%. This indicates that the reactions with N₂ were saturated with hydrolytic enzymes, while the reaction catalyzed by LPMO created new substrates for hydrolytic enzymes in a synergistic manner, so that saturation did not occur.

Different structural analyses were performed to characterize the substrates further. Structural analyses and surface properties of the solid fraction were performed by using different analytical techniques. Characterization of surface properties based on BET analysis showed that the surface area increased with 79% to 112% in the reactions with air, and with 20% to 31% in reactions with N₂. Simons' staining resulted in higher DO dye values for the reactions with air than for reactions with N₂ (Fig. 3B in Paper II), suggesting improved cellulose accessibility for cellulase enzymes, as the DO dye has been found to have affinity for cellulose rather than for hemicellulose and lignin (Chandra and Saddler, 2012). In addition, Arantes and Saddler (2011) suggested that the accessible surface area was one of the factors that limit the extent of enzymatic saccharification of cellulose. This can explain the results we observed that the reactions with air continuously showed increasing saccharification even when the dosage was raised from 9% to 12%, while reactions with N₂ seemed to be saturated. LPMO has potential to improve enzymatic saccharification through its ability to oxidatively

cleave glycosidic bonds to create more targets for hydrolytic enzymes to work on, especial with respect to targeting more crystalline parts of the cellulose, thus improving the efficiency of the saccharification. The results from the XRD and SEM analyses also point in the same direction. For instance, XRD measurements showed a clearer decrease in crystallinity for reactions with air than for reactions with N₂ (Table 3 in Paper II), and obvious structural changes were observed using SEM (Fig. 6 in Paper II). In summary, the investigation highlights the great potential in using continuous aeration to enhance the LPMO activity in reactions with high solids loadings.

Comparison of process configurations (Paper III)

Saccharification and fermentation can be performed using different process configurations (Galbe and Wallberg, 2019; Gandla et al., 2022; Martín et al., 2022). Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) have been studied for a long time. Both process configurations have their advantages and disadvantages. SSF has been considered a good option with the older generation of cellulases preparations, as it typically results in higher yields of ethanol (Erdi et al., 2010; Tomás-Pejó et al., 2008).

Inclusion of LPMO in modern enzyme preparations may, however, affect the choice of process configuration (Cannella and Jørgensen, 2014). Hybrid hydrolysis and fermentation (HHF) is a potential alternative, which was studied more in detail in the investigation presented in Paper III. HHF involves an enzymatic pre-hydrolysis phase performed at higher temperature, which is preferable for the enzymatic reaction, while the fermenting microorganism, typically yeast, is added afterward. Additionally, oxygen can be supplied by using aeration during the pre-hydrolysis phase in order to improve the enzymatic saccharification by driving the activity of LPMO. In Paper III, HHF was

compared with SSF by using an LPMO-containing enzyme preparation and a modern, commercial xylose-utilizing yeast strain. The studies were conducted in a demonstration plant under industrial-like conditions. Two different aeration time periods were compared: HHF1 (24 h) and HHF2 (48 h). There was no aeration in the SSF, but on the other hand no measures were taken to get rid of oxygen in the headspace of the bioreactor.

According to the data shown in Table 2 in Paper III, the glucose concentrations increased as expected during the initial saccharification phase. Although the glucose increased quickly in HHF2 within the first 24 h, similar to the HHF1 reaction, there was little change between 24 h and 48 h. This might suggest a possible negative effect from end-product inhibition becoming increasingly worse. The increased glucose concentrations in the early stages of the HHF runs could be partially attributed to aeration and LPMO activity. This is supported by data for oxidation products (Fig. 2 in Paper III), which clearly show formation of oxidation products during saccharification. The glucan conversion data (Fig. 1 in Paper III) show that after 24 h and 48 h the conversion in the HHF1 and HHF2 reactions was more than twice as high as for SSF, and after 96 h the glucan conversion had reached roughly 70% of the conversion in HHF. This might be explained by the higher temperature and the continuous aeration in the pre-hydrolysis phase was beneficial for enzymatic saccharification including LPMO activity, thus contributing to the higher glucan conversion. However, after pre-hydrolysis the temperature was lowered and the aeration was discontinued. This is why the conversion in the SSF reaction was able to catch up after 96 h.

Based on the ethanol results (Table 4 in Paper III), SSF consistently showed the highest volumetric ethanol productivity, reaching 0.43 g/L/h, whereas HHF1 exhibited moderate productivity (~0.3 g/L/h) and HHF2

very low productivity (around 0.1 g/L/h). In addition, the ethanol yield on consumed sugar in the SSF reaction was consistently higher than in the HHF reactions, both after 24 h and 48 h fermentation time. The low ethanol yields on consumed sugar might be due to that the sugar was used for other purposes than for ethanolic fermentation. *S. cerevisiae* is a Crabtree-positive yeast that still can produce ethanol even under aerobic conditions, when sugar concentrations are sufficiently high. However, considering the large headspace in the bioreactor and the continuous aeration, there is a risk that the sugar is used for other purposes than for ethanol production. Furthermore, the fermentation results after addition of yeast to the HHF reactions were so dismal that it triggered the investigation presented in Paper IV, which addresses the possibility that the fermentability might, at least in some cases, become poorer as a result of aeration.

Effects of aeration on inhibitors and fermentability (Paper IV)

Incorporating LPMOs into enzyme cocktails significantly improves saccharification efficiency, particularly under continuous aeration, which enhances LPMO activity (Tang et al., 2022). However, the introduction of LPMOs has led to reconsiderations of the process configurations used for saccharification and fermentation processes (Cannella and Jørgensen, 2014). A hybrid hydrolysis and fermentation (HHF) approach using LPMO-enriched enzymes has been explored, involving a pre-hydrolysis step prior to yeast addition (Tang et al., 2024). Although aerated pre-hydrolysis with LPMO as expected improved the saccharification efficiency, it unexpectedly resulted in poor fermentability (Tang et al., 2024). Aeration may not only promote LPMO activity but may also affect pretreatment by-products. One possibility is

oxidation reactions caused by aeration, and another possibility is evaporation caused by introducing a gas flow.

Detoxification methods based on redox reactions were also included in this study. These were treatments with reducing agents (Jönsson and Martín, 2016) and with the phenol oxidase laccase (Jönsson et al., 1998; Ibarra et al., 2023). Additionally, laccase and hydroquinone were included in some experiments. Although hydroquinone in low concentrations is relatively non-toxic to yeast (Larsson et al., 2000; Mitchell et al., 2014), it can be oxidized to benzoquinone, a highly toxic compound even at low concentrations (Stagge et al., 2015; Martín et al., 2018). However, hydroquinone may also act as a reductant, potentially mitigating inhibition. To further investigate the effects of aeration, parallel experiments were conducted using both air and N₂.

By comparing the effects of gas addition to PL and a sugar control, significant differences were observed between aeration and N₂ for PL, while there were no differences for the sugar control (Fig. 1 in Paper IV), and a better fermentation was obtained for the N₂-treated set of reaction mixtures (Table 2 in Paper IV). Potential changes in the concentrations of pretreatment by-products are reported in detail in Table 3 in Paper IV. Furan aldehydes decreased significantly, both with aeration and with N₂, with furfural showing a larger decrease than HMF due to its higher volatility. The acetaldehyde concentrations also decreased significantly for both air and N₂. Among the aromatic compounds, the vanillin concentrations showed a slight but significant decrease both for air ($p \leq 0.05$) and for N₂ ($p \leq 0.01$). This is likely due to its volatility resulting in evaporation during gas addition.

Detoxification with sulfite significantly enhanced the fermentability compared to controls without sulfite (Table 2 in Paper IV). Higher sugar consumption and ethanol production rates were consistently observed for aerated reactions compared to reactions with N₂ without sulfite,

demonstrating that sulfite detoxification was effective regardless of how the aeration was done. For specific inhibitors, detoxification was particularly effective in reducing formaldehyde and coniferyl aldehyde concentrations to 1.3 mM and 15 μ M, respectively, compared to 5.6 mM formaldehyde and 26 μ M coniferyl aldehyde before detoxification.

Spiking with hydroquinone before aeration resulted in a significant improvement of the fermentation rates compared to the N₂ controls (Table 2 in Paper IV). This suggests that hydroquinone acts as an effective detoxification agent in the presence of inhibitors, because there were no differences between air and N₂ conditions in the control experiment. Additionally, aeration resulted in a slight increase in the formaldehyde concentrations, indicating a minor but statistically significant ($p \leq 0.05$) rise under aeration conditions, something that was not observed with N₂ treatment.

The presence of catalysts, such as the phenol oxidase laccase, can significantly enhance the effects of oxygen. Laccase treatment has been demonstrated to improve the fermentability of lignocellulosic hydrolysates by targeting phenolic substances (Jönsson et al., 1998; Ibarra et al., 2023). However, there is also a risk, at least theoretically, that laccase could oxidize hydroquinone into the more toxic benzoquinone. The experiments where hydroquinone was added before incubation, compared with the controls without any additions, confirmed that adding hydroquinone at a concentration of 1 mM was effective as a detoxification method (Table 5 in Paper IV). This aligns with findings from previous experiments with spiking with hydroquinone. Adding hydroquinone after incubation showed some improvement compared to a control without any additions, but the effect was significantly less than when hydroquinone was added before incubation. Additionally, laccase treatment performed worse than the control without additions. The total phenolic content decreased from

around 2.3 g/L in the untreated PL to around 1.0 g/L with laccase, indicating a clear reduction in phenolic content - a typical detoxification reaction. However, as the PL is a complex mixture, it may contain inhibiting compounds that are more toxic than phenolics. Using a system with synthetic medium without PL, it was evident that laccase catalyzed the oxidation of hydroquinone to a more toxic compound, presumably benzoquinone (Fig. 3 in Paper IV), and that hydroquinone was not inhibitory in the beginning due to the relatively low initial concentration (1 mM). The effect of laccase treatment is mixed as it may alleviate or intensify inhibition depending on the composition and concentration of inhibitors in the pretreated biomass.

Future perspectives

The effectiveness of LPMO in a commercial enzyme mixture, combined with oxidants and reductants, has been demonstrated in the enzymatic saccharification of softwood pretreated by continuous steam explosion with sulfur dioxide as a catalyst. Experiments with continuous air and N₂ supply revealed that both insoluble lignin in the solid phase and water-soluble lignin fragments in the liquid phase act as effective reductants in LPMO-supported saccharification. The harsh pretreatment conditions resulted in a solid phase predominantly containing Klason lignin, cellulose, and pseudo-lignin, and the liquid phase was rich in lignin-derived phenolics. Nevertheless, continuous aeration promotes LPMO activity even if there are relatively high concentrations of by-products in the liquid phase and pseudo-lignin in the solid phase. It remains to identify specific degradation products and structures that serve as the reductants to improve the LPMO activity. Given the high complexity of pretreatment mixtures, further investigations using a pure system could offer a good option to provide more detailed insights.

According to the negative effect associated with high solids loadings, continuous aeration in the LPMO-supported saccharification showed clear benefits. In addition, the effects of increasing enzyme dosage were not as efficient as applying aeration into the reaction. The applicability of these findings can be further investigated using other feedstocks. Due to the relatively harsh pretreatment, milder pretreatment will be an option.

According to the study on process configuration, hybrid hydrolysis and fermentation (HHF) with pre-hydrolysis under continuous aeration exhibited a positive effect on glucan conversion. Extending the reaction time from 24 to 48 hours further improved saccharification but had even

worse effect on subsequent microbial fermentation. Positive outcomes include that aeration can reduce volatile inhibitors, like furfural, through evaporation. To mitigate inhibitory effects, incorporating of a detoxification step prove to be beneficial and sodium sulfite emerged as an effective reducing agent targeting formaldehyde, while hydroquinone appears as a previously unknown alternative for alleviating inhibition. As a relatively harsh pretreatment was performed using a recalcitrant form of lignocellulose, i.e., softwood, using milder pretreatment conditions and studying other feedstocks are interesting options for future research in the field. Furthermore, techno-economic assessment will be needed for analyzing the technical and economic feasibility of the process before industrial-scale deployment.

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