Norwegian University of Life Sciences
Faculty of Chemistry, Biotechnology and Food Science
Department of Bioprocess Technology and Biorefining

Philosophiae Doctor (PhD)
Thesis 2022:29

Leveraging H_2O_2-fuelled activity of lytic polysaccharide monooxygenases in cellulase cocktails for improved bioprocessing of lignocellulosic biomass

Utnyttelse av H_2O_2-drevet lytisk polysakkarid monooksygenase-aktivitet i cellulaseblandinger for forbedret bioprossesering av lignocelluloseholdig biomasse

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Summary

Rapid action is critical to reduce anthropogenic CO_2 emissions in order to reach climate targets. Fossil fuel use in high emission sectors like the energy and chemical industry should therefore be reduced and replaced by sustainable alternatives. Lignocellulosic biomass, like Norwegian spruce, has potential to replace commonly used feedstocks like corn and sugarcane in a biorefinery for production of biofuels and bio-based chemicals due to its high abundance, high proportion of polysaccharides, and limited competition for arable land. Lignocellulose is a highly recalcitrant feedstock that requires high severity pretreatment and enzyme loads to produce fermentable sugars. The inclusion of lytic polysaccharide monooxygenase (LPMO) to cellulase cocktails has previously been shown to enhance saccharification yields considerably. Recent findings that LPMO activity is driven by H_2O_2 opens the door to novel process setups to fully harness this potential. The aim of this thesis was to improve the conversion of lignocellulosic biomass by altering process conditions to maximize the impact of LPMOs in saccharification and fermentation processes.

In **Paper 1**, recent developments within enzymatic processing of lignocellulosic biomass are summarized, and we show the interconnectedness between pretreatment and saccharification, and how this affects LPMOs in the cellulase cocktail. The implications of direct addition of H_2O_2 compared to *in-situ* generation is discussed in relation to choice of feedstock and process design.

The results in **Paper 2** demonstrate how negative impacts from residual lignin in the saccharification step can be alleviated by inclusion of the carbocation scavenger 2-naphthol in a steam explosion pretreatment. Lignin resulting from 2-naphthol-impregnated samples improved LPMO activity not only in the cellulase cocktail but also of the chitin-active LPMO CBP21 (*Sm*AA10A), showing that more H_2O_2 could be generated in the presence of impregnated lignin. This paper also explores a potential combination of biochemical (i.e., enzymatic saccharification) and thermochemical (i.e., pyrolysis) conversion steps for a softwood-based biorefinery. Impregnation had no negative impacts on bio-oil produced by pyrolysis.

In **Paper 3**, direct addition of H_2O_2 to separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes was used to boost

LPMO activity in a commercial enzyme cocktail. Production of lactic acid by *B. coagulans* in an SSF setup was found to be competitive with the SHF process in this study contrary to previous findings. In particular, feeding H_2O_2 during SSF enabled oxidative cellulose depolymerization by LPMOs present in a cellulase cocktail under anaerobic conditions.

Overall, the findings presented in this thesis illustrate how latent potential of LPMOs in commercial cellulase cocktails can be utilized to improve existing saccharification and fermentation processes. This is particularly relevant for bioprocessing of recalcitrant lignocellulosic feedstocks like the softwood Norway spruce.

Sammendrag

For å redusere menneskeskapte CO_2-utslipp og for å nå klimamålene er det avgjørende å handle raskt. Bruk av fossilt brensel i høyutslippssektorer som energi- og kjemisk industri bør derfor reduseres og erstattes av bærekraftige alternativer. Lignocelluloseholdig biomasse, som for eksempel norsk gran, kan potensielt erstatte vanlig brukte råvarer som mais og sukkerrør i et bioraffineri for produksjon av biodrivstoff og biobaserte kjemikalier på grunn av den finnes i store mengder, har høyt innhold polysakkarider og i liten grad konkurrerer om dyrkbar jord. Lignocellulose er et svært gjenstridig råmateriale som krever intens forbehandling og høy enzymbelastning for å produsere fermenterbare sukker. Å inkludere lytisk polysakkarid monooksygenaser (LPMOer) i cellulaseblandinger har tidligere vist seg å øke sukkerutbyttet betraktelig. Nye oppdagelser som viser at LPMO-aktivitet er drevet av H_2O_2 muliggjør nye prosessoppsett som kan utnytte dette potensialet fullt ut. Målet med denne oppgaven var å forbedre omdannelsen av lignocelluloseholdig biomasse ved å endre prosessforhold for å maksimere virkningen av LPMO i sukkerfrigjørings- og fermenteringsprosesser.

I **Artikkel 1** er nyere utviklinger innen enzymatisk prosessering av lignocelluloseholdig biomasse oppsummert, og vi viser sammenhengen mellom forbehandling og sukkerfrigjøring, og hvordan dette påvirker LPMOer i cellulaseblandingen. Betydningen av direkte tilsetning av H_2O_2 sammenlignet med in-situ dannet diskuteres i sammenheng med valg av råstoff og prosessdesign.

Resultatene i **Artikkel 2** viser hvordan negative påvirkninger fra gjenværende lignin i sukkerfrigjøringstrinnet kan lindres ved å inkludere karbokationfangeren 2-naftol under en dampeksplosjonsforbehandling. Ligninet ga som følge av 2-naftolimpregneringen forbedret LPMO-aktivitet ikke bare i cellulaseblandingen, men også for den kitinaktive LPMOen CBP21 (SmAA10A), noe som viser at mer H_2O_2 kunne dannes med impregnert lignin til stede. Denne artikkelen utforsker også en mulig kombinasjon av biokjemiske (dvs. enzymatisk sukkerfrigjøring) og termokjemiske (dvs. pyrolyse) konverteringstrinn for et bartrebasert bioraffineri. Impregnering hadde ingen negativ innvirkning på bioolje produsert ved pyrolyse.

I **Artikkel 3** ble direkte tilsetning av H_2O_2 anvendt i separate hydrolyse- og fermenteringsprosesser (SHF) og samtidige sukkerfrigjørings- og fermenteringsprosesser (SSF) for å øke LPMO-aktiviteten i en kommersiell enzymblanding. Produksjon av melkesyre med *B. coagulans* i et SSF-oppsett var konkurransedyktig med SHF-prosessen i denne studien, i motsetning til tidligere funn. Særlig viktig var det at tilsats av H_2O_2 under SSF muliggjorde oksidativ cellulosedepolymerisering ved bruk av en cellulasblanding som inneholdt LPMOer under anaerobe forhold.

Funnene presentert i denne oppgaven illustrerer hvordan det latente potensialet til LPMOer i kommersielle cellulaseblandinger kan utnyttes bedre for å forbedre eksisterende sukkerfrigjørings- og fermenteringsprosesser. Dette er spesielt aktuelt for bioprosessering av gjenstridige lignocelluloseholdige råstoffer som bartreet gran.

Abbreviations

1G	First generation
2G	Second generation
3G	Third generation
4G	Fourth generation
5-HMF	5-Hydroxymethylfurfural
BECCS	Bioenergy combined with carbon capture and storage
BG	β-glucosidase
CBH	Cellobiohydrolase
CBM	Carbohydrate binding domain
CBP	Consolidated bioprocessing
CCS	Carbon capture and storage
CDR	Carbon dioxide removal
CE	Carbohydrate esterase
EG	Endoglucanase
GH	Glycoside hydrolase
GHG	Greenhouse gas
Glc4gemGlc	4-Hydroxy-β-D-xylo-hexopyranosyl-(1→4)-β-D-glucopyranosyl
LAB	Lactic acid bacteria
LCC	Lignin carbohydrate complexes
LPMO	Lytic polysaccharide monooxygenase
PDLA	Poly-D-lactic acid
PE	Polyethylene
PLA	Polylactic acid
PLLA	Poly-L-lactic acid
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
SSNMR	Solid state nuclear magnetic resonance spectroscopy
XOS	Xylo-oligosaccharides

List of papers

Paper 1:

Østby, H., Hansen, L.D., Horn, S.J., Eijsink, V.G.H, Várnai, A. (2020). "Enzymatic processing of lignocellulosic biomass: principles, recent advances and perspectives." Journal of Industrial Microbiology & Biotechnology **47**: 623-657.

Paper 2:

Hansen, L. D., Østensen, M., Arstad, B., Tschentscher, R., Eijsink, V.G.H., Horn, S.J., Várnai, A. (2022). "2-Naphthol impregnation prior to steam explosion promotes LPMO-assisted enzymatic saccharification of spruce and yields high-purity lignin." ACS Sustainable Chemistry & Engineering **10**: 5233-5242.

Paper 3:

Hansen, L.D., Eijsink, V.G.H., Horn, S.J., Várnai, A. "H_2O_2 feeding enables LPMO-assisted cellulose saccharification during simultaneous fermentative production of lactic acid." Submitted to: Biotechnology and Bioengineering

Other papers by the author involving lignocellulosic biomass and fermentation:

Sharma, S., Hansen, L. D., Hansen, J. Ø., Mydland, L. T., Horn, S. J., Øverland, M., Eijsink, V.G.H., Vuoristo, K. S. (2018). "Microbial protein produced from brown seaweed and spruce wood as a feed ingredient." Journal of Agricultural and Food Chemistry **66:** 8328-8335.

Lapeña, D., Kosa, G., Hansen, L. D., Mydland, L. T., Passoth, V., Horn, S. J., & Eijsink, V. G. (2020). "Production and characterization of yeasts grown on media composed of spruce-derived sugars and protein hydrolysates from chicken by-products." Microbial Cell Factories **19:** 1-14.

1 Introduction

1.1 A global perspective on CO_2 emissions

It is a fact that release of greenhouse gasses (GHGs) like carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) from human activity directly impacts the planet's climate, based on new findings from the recently released IPCC report (Arias et al., 2021). Estimates now predict that the mean temperature of the Earth's surface will increase between 1.5 and 2°C within the next century compared to 1850-1900, with the years 2016-2020 being the hottest period on record. It is estimated that we will already cross 1.5°C in the early 2030s.

A variety of compounds released by human activity participate to this increase, but it is mainly driven by release of CO_2 into the atmosphere. In contrast to short-lived climate forcers like CH_4 and aerosols that decay within a few decades following emission, CO_2 is more stable in the atmosphere and is mostly responsible for long-term warming effects. Due to the close historical relationship between global CO_2 emission levels and measured warming, CO_2 emission levels have proven to be an effective predictor of global warming. Legislature therefore often emphasizes CO_2 emission reduction goals as a part of their action strategy.

An increase in atmospheric CO_2 from increased emissions leads to more accumulation of CO_2 in land and oceans as these act as carbon sinks (Figure 1). Acidification of most of the deep oceans, as a result of this, has already been established with a global decline in pH of the ocean surface in the past four decades (Arias et al., 2021). Currently, atmospheric CO_2 concentrations are at the highest level in 2 million years. If CO_2 emissions keep increasing during this century, less and less CO_2 can be taken up by carbon sinks.

In addition to land and ocean being carbon sinks, CO_2 can be removed from the atmosphere by carbon dioxide removal (CDR) methods, which include direct carbon capture. The most promising CDR strategies involve restoration of natural local ecosystems or improved sequestration of carbon in soil (Arias et al., 2021). Since CO_2 is released from carbon sinks simultaneously with CO_2 being captured, to reduce global CO_2 levels, more CO_2 must be removed than is emitted. However, CDR does not address

side effects occurring from high levels of CO_2 stored on land and water including ocean acidification and detrimental effects on food production and biodiversity.

Mitigating effects such as release of aerosols into the stratosphere, dissolution of cirrus clouds and marine cloud brightening to cause cooling effects, only produce short term local effects and are not suitable to counteract the warming caused by high release of CO_2. These methods also do not address other issues like ocean acidification.

For the highest probability (Shared Socioeconomic Pathway 1 (SSP1)) of limiting global warming to 1.5°C, net zero carbon emissions must be reached by 2050 (Rogelj et al., 2018). This means necessary change from high emission countries and industries at an unprecedented level (Riahi et al., 2015). Historically, the majority of CO_2 released into the atmosphere results from the energy sector. In 2021, CO_2 emissions from the energy sector rose by 6% to 36.3 Gt, replacing 2010 as the highest annual increase on record (IEA, 2022b), even rising above pre-pandemic levels. This is partly due to burning of coal for energy generation due to high natural gas prices. Within the energy sector in the EU, transport makes up 29% of CO_2 emissions distributed among passenger cars and vans (15%), heavy duty vehicles (5%), aviation (4%), maritime (4%) and light vehicles and rail transport (1%).

Even though electrification of passenger cars has led to a reduction in CO_2 emissions, there is still a need for liquid fossil fuels for heavy duty vehicles like trucks and in the aviation sector. There is therefore a need to lower CO_2 emissions from these liquid fuels in addition to overall reduction to meet net zero CO_2 emission goals.

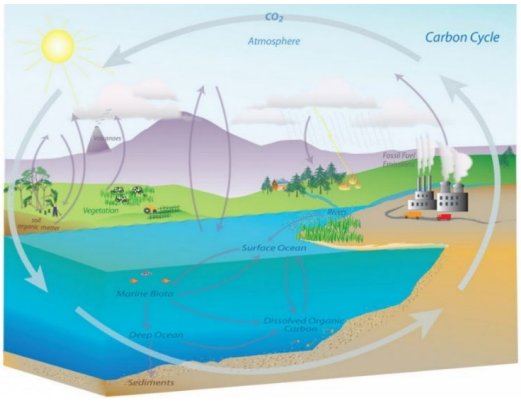

Figure 1 – Schematic view of the carbon cycle. Upward arrows indicate CO_2 release, downward arrows indicate CO_2 capture. Figure from NOAA (2019)

1.2 Reducing CO_2 emissions

Fossil fuel use, which accounted for 79% of global energy demand in 2020 (IEA, 2021b), must be reduced within the next century to limit global warming to 1.5°C (Rogelj et al., 2018). It is estimated that 60% of oil and fossil methane gas and 90% of coal reserves must remain unextracted by 2050 compared to 2018 reserves (Welsby et al., 2021). To achieve this, there must be a global decrease in oil and gas production of 3% every year until 2050. Increased emissions from fossil fuels have led to increasing CH_4 emissions from 2014-2019. CH_4 emissions from fossil fuel sources are particularly problematic as there is concurrent release of CO_2 in contrast to biogenic CH_4 emissions (Arias et al., 2021). To reduce the dependency on fossil fuel use in the petrochemical sector, it is necessary to reduce the global consumption of oil-based products like plastics (Lau et al., 2020).

1.2.1 Fuels

The largest portion of anthropogenic CO_2 emissions in the last decade (86%) has come from combustion of fossil fuels. (Arias, 2021). The fastest growing sector in terms of GHG emissions is the transport sector, where a majority of emissions stem from passenger road transport (Kawamoto et al., 2019).

Electrification of vehicles combined with a clean energy supply significantly decreases CO_2 emissions and is seeing increased adoption for passenger cars. In 2021, the amount of electric cars sold globally doubled to 6.6 million, representing 9% of the global car market (IEA, 2022a). However, a 10% increase in sales of internal combustion engine SUVs during the same period offset CO_2 reductions by the electric car fleet (IEA, 2021a), highlighting the need for a greener liquid fuel blend.

Use of non-carbon fuels like hydrogen and ammonia are both promising technologies to replace fossil fuel use in internal combustion engines (Erdemir & Dincer, 2021; Singla et al., 2021) and eliminate storage issues associated with clean electricity. Freight transport, the petrochemical industry, and the aviation industry are more challenging to decarbonize. Fossil fuel use in these sectors can still be expected after 2050 (Luderer et al., 2018), barring a major technological breakthrough or change in consumer demand.

The use of biomass for bioenergy is the main driver of reduction of carbon intensity in fuels (Luderer et al., 2018). The amount of extractable fossil fuel reserves is highly influenced by future availability of biomass that can be used for bioenergy (Welsby et al., 2021). Bioenergy from biomass has potential to be a negative emission technology when combined with carbon capture and storage (CCS) as described below. It is however necessary to minimize sustainability issues with use of biomass for bioenergy such as indirect land use change and competition with growth of food crops (Daioglou et al., 2016). This is compounded by increasing food demand in the next century (van Vuuren et al., 2017) and a potential decrease in arable land.

In the most aggressive CO_2 reduction estimate, biomass will account for 86% of global use of solid final energy for industry and building sectors in 2050 and 28% of liquid fuels for transportation, with a high proportion being biofuels for trucks and planes (Luderer et al., 2018). Biofuels is a broad term referring to bioethanol and -methanol, biodiesel, biooil, bio-syngas, Fischer-Tropsch liquids, biochar, biomethane and biohydrogen (Balat, 2011). Biofuels can be produced by a variety of processes including: biochemical conversion, pyrolysis, gasification, liquefaction, and supercritical fluid/water extraction (Balat, 2011). Bioethanol makes up the vast majority of biofuels used worldwide. It can be blended with regular petrol at 5-10% levels and used directly in current internal combustion engines, with modified engines able to run on blends containing 15-20% bioethanol (Balat, 2011).

The period 2020-2030 is critical and will determine how much we will rely on mitigating factors to achieve net zero CO_2 emissions by 2050 (Riahi et al., 2015). It is necessary to invest in and upscale low-carbon technologies like bioenergy from biomass to avoid further investment into fossil fuel infrastructure, making it harder to transform the industry. Without this, technology developments will be too slow to outcompete oil (van Vuuren et al., 2017). The lifetime for infrastructure in the energy sector is 30-60 years (Riahi et al., 2015), which offers little flexibility to introduce new technology. It is critical that low-carbon technologies are scaled up and fossil fuel power plants are phased out – even if that means premature closure of plants (Riahi et al., 2015).

1.2.2 Carbon dioxide removal

Even with reduction of global use of fossil fuel, it is unlikely that this will be sufficient to meet zero emission goals, and it will be necessary to simultaneously employ CDR measures (Luderer et al., 2018).

A promising negative emission technology is bioenergy combined with carbon capture and storage (BECCS). In BECCS, atmospheric CO_2 is taken up by growing biomass, and released CO_2 from bioenergy combustion is captured and stored underground (Anderson & Peters, 2016). CO_2 can be captured from the flue gas stream resulting from combustion of biomass and waste streams or produced directly from fermentation in a biorefinery (Jones & Albanito, 2020). The only large-scale plant utilizing this technology is found in Illinois and captures carbon from bioethanol fermentation using corn (a 1st generation feedstock) as the starting material (Consoli, 2019). The viability of CCS has not been proven for lignocellulosic biomass (2nd generation feedstocks). BECCS also suffers from sustainability issues associated with use of bioenergy, and future potential could be limited by reduced biomass availability (Riahi et al., 2015).

Heavy reliance on CDR measures such as negative emission strategies like BECCS is often favored by policy makers since it reduces the need for immediate climate action and rather assumes that tomorrow's technologies will be readily available to solve today's problems. This stifles critical rapid action and allows for continued use of fossil fuels (Anderson & Peters, 2016). CDR measures are a necessity to achieve net zero CO_2 emissions by 2050, but their use is limited and will not be sufficient to solve problems associated with current continued emissions. If the promise of CCS technology is never realized, it severely increases the cost of future mitigation efforts, and it becomes infeasible to achieve CO_2 reduction targets in 75% of model predictions (Riahi et al., 2015), highlighting how reliant prediction models are on the maturity of this technology. The second largest impact was observed if availability of biomass becomes limited (Riahi et al., 2015). Biomass and CCS will therefore be critical to any policies that rely on negative CO_2 emissions.

1.2.3 Plastics

In addition to applications for production of biofuels and in CCS approaches, 2nd generation biomass has the potential to transform the plastics industry. Residues from plastic pollution can be found in rivers, oceans, animal biomass, and the atmosphere (Lau et al., 2020). The current volume of plastics produced leads to unmanageable levels of plastic waste, which results in unsustainable waste management strategies like burning. Burning plastics is the largest contributor to waste mismanagement and results in release of microplastic particles and toxic air pollutants, in addition to GHGs, which are mostly impacting the local area (Wiedinmyer et al., 2014; Zheng & Suh, 2019). This problem is exacerbated by displacing plastic waste globally, with importing countries often not having enough capacity to handle this exogenous waste (Velis, 2015).

The entire plastic production chain including production (accounts for most emissions), collection of waste and waste disposal is responsible for GHG emissions (Zheng & Suh, 2019). If plastic consumption patterns are not altered, mismanaged plastic waste will roughly double by 2050 compared to 2015 levels (Geyer et al., 2017).

Recycled plastics have lower GHG emissions during their lifetime (Zheng & Suh, 2019), but composite products like multi-material plastics are difficult to recycle. Only about 40% of collected plastics are recycled, and most can only be recycled once (MacArthur et al., 2016). Additionally, most plastics are not collected or properly separated. In total, only 14% of plastics were recycled globally in 2013 (MacArthur et al., 2016). To reduce GHG emissions from the plastic industry, a combined and coordinated approach is therefore necessary where overall production of virgin plastic is decreased and partially replaced by bio-based alternatives while waste management is similarly improved (Lau et al., 2020; Zheng & Suh, 2019).

Plastics for packaging accounts for 26% of the total volume of plastic produced (MacArthur et al., 2016) and has a short lifetime – often being used within a year of production. The widespread use of plastic for packaging applications is due to superior barrier properties which help prolong shelf-life of foods and reduce food waste. The light weight of plastic packaging also reduces fuel consumption during its transport (MacArthur et al., 2016), however, this is offset by the high volumes of plastics produced and the high GHG emission built into their lifecycle.

Currently, the vast majority of plastics are produced from the monomers ethylene and propylene, both of which result from fossil hydrocarbons (Geyer et al., 2017). In total 90% of the feedstock for the plastic industry originates from oil and gas (MacArthur et al., 2016). If we consider fossil fuel use in production plants, consumption of oil by the plastics industry is on the same scale as use by the aviation sector globally (IEA, 2014; MacArthur et al., 2016) and is expected to increase within the next century if no measures are taken, even outpacing total demand for oil (IEA, 2015).

To reduce reliability on fossil fuels in the plastic industry, it is not enough to strengthen reduction and recycling efforts. A part of the solution will involve the replacement of virgin oil-based plastics with more sustainable options (MacArthur et al., 2016). Greener plastic alternatives can be either bio-based, compostable (biodegradable) or both. It is possible to produce chemically identical plastics like polyethylene terephthalate and polyethylene (PE) from renewable sources (MacArthur et al., 2016), which can directly substitute their fossil fuel derived counterparts. However, these will suffer from the same issues in terms of end-of-life treatment. The second group of green plastics like polylactic acid (PLA), thermoplastic starch and polyhydroxyalkanoates are compostable but have novel properties and will require new infrastructure both for production and end-of-life treatment. An advantage of using compostable plastic for food packaging is the potential for residual food in the container to enter compost which returns valuable organic nutrients to the soil. If compostable plastic is to take up more of total plastic in circulation, collection and separation of plastics must improve to reduce cross-contamination with traditional plastic (Zheng & Suh, 2019).

PLA is a promising environment-friendly alternative to oil-based plastics (Nofar et al., 2019). Lactic acid can be polymerized into either poly L-lactic acid (PLLA) and poly D-lactic acid (PDLA) depending on the starting enantiomer. Mixes of L-lactic acid and D-lactic acid can also be polymerized to yield biopolymers with altered properties like melting point, tensile strength, and degradability (Nofar et al., 2019). PLLA is semi-crystalline and has the largest potential to replace plastics in packaging applications (John et al., 2007). PLA plastics are still twice as expensive as PE, and their production process should be optimized to increase market share.

1.2.4 The biorefinery concept

Bioethanol and PLA are both potential products of a biorefinery. A biorefinery, much like a traditional oil refinery, is used to fractionate biomass and produce fuels and chemicals. In a biorefinery, biomass replaces crude oil as the starting material. A key aspect of the biorefinery is the conversion of biomass to platform sugars that can be fermented to produce a wide array of end products (Figure 2). Due to its high availability and low-cost, lignocellulose is an attractive feedstock for biorefinery applications.

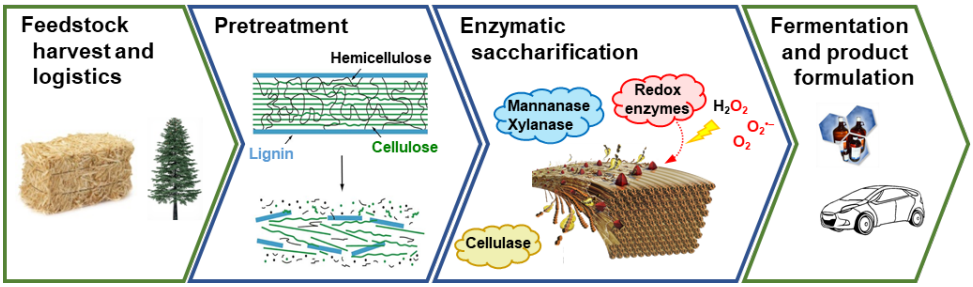

Figure 2 – An overview of the biorefinery process. In the pretreatment step, the lignocellulosic matrix is disrupted, and accessibility of the cellulose is increased. During enzymatic saccharification of the pretreated materials, a cellulase cocktail depolymerizes polysaccharides and produces a liquid fraction rich in fermentable sugars that can be used in the fermentation step to produce a variety of end products like bioethanol or lactic acid for bioplastic production. Figure from Østby et al. (2020).

An overview of potential feedstocks for biofuel production can be found in Table 1. First generation (1G) feedstocks are rich in sucrose and starch. Feedstocks like corn, wheat, and sugarcane are used commercially in the US, Europe, and Brazil, respectively, (Balat, 2011) for production of bioethanol, which can be blended with fuels to lower CO_2 emissions. However, since these directly compete with food supply, second generation (2G) feedstocks are considered more suitable for future bioethanol and bio-based product manufacturing. 2G sources include wood, forest- and crop-residues, waste paper, and municipal solid waste (Balat, 2011). Third generation (3G) sources are lipid-producing microalgae for biooil production (Rajesh Banu et al., 2020); and fourth generation (4G) biofuel technology builds on microalgae cultivation, but relies on metabolically engineered strains that can grow in photobioreactors with increased yields and CO_2 utilization (Moravvej et al., 2019). 1G and 2G biorefineries both rely on utilization of sustainably produced platform sugars in downstream fermentation processes and can be used to produce bio-based chemicals in addition to biofuel.

Table 1 – An overview of biorefinery feedstocks and technology for biofuel production. Based on information from Dutta et al. (2014).

	Feedstock	Main polysaccharide	Biofuel product	Benefits	Limitations
1G	Food crops	Sucrose, starch	Bioethanol	Economical process	Competition with food supply
2G	Wood, forest- and crop-residues, municipal solid waste	Cellulose, hemicellulose	Bioethanol	Create value from waste, no competition with food	Expensive pretreatment and saccharification
3G	Microalgae	-	Biodiesel	Simple cultivation	Contamination issues in open pond systems, low yields
4G	Modified photosynthetic microorganisms	-	Biodiesel	Enhanced productivity, increased CO_2 capture	Low technology readiness level (TRL), high cost

An appropriate feedstock for a biorefinery will need to have a high land-use efficiency, a low water footprint, no competition with food crops and should minimize environmental impact, for example from increased emissions from indirect land use change or increased use of fertilizer (Azadi et al., 2013). In Norway, 37% of the land area is covered by forest, making up a total area of 12 million ha (Scarlat et al., 2011). Downy birch is the most abundant tree species, but the majority of biomass available is the softwood Norway spruce (*Picea abies*) (Breidenbach et al., 2021). Firewood and residues from the logging industry are the main biomass resources extracted from the Norwegian forest (Scarlat et al., 2011). Norway spruce therefore makes up an attractive candidate for a lignocellulose biorefinery in Norway. Barriers for economic production of bioproducts from 2G feedstocks in a biorefinery are storage and transportation of the low density feedstocks, the recalcitrant nature of the feedstock, and finding a suitable resistant strain that can metabolize all sugars (Balat, 2011). To overcome these economic barriers, it is necessary to reach high conversion at low cost. Cost reduction starts at the beginning of the conversion process; optimizing pretreatment and saccharification steps are the necessary first steps towards the realization of a 2G biorefinery.

1.3 Lignocellulosic biomass

Lignocellulose is the most abundant biomass found in nature. It is widely available from forestry and agricultural sources as well as waste from the agroindustry (Duff & Murray, 1996). Primary waste sources are direct by-products of the agroindustry like wheat straw and corn stover as well as residues from the milling process in the form of sawdust and bark. Secondary waste sources are local, municipal, and commercial waste sludge which contains lignocellulose in the form of paper, cloth, and garden waste (Duff & Murray, 1996).

The plant cell wall provides support and structure to plants, allowing growth of up to 100 meters (Scheller & Ulvskov, 2010). Additionally, the plant cell wall forms a protective barrier against the environment, while also allowing for expansion during cell growth and exchange of materials between cells (Scheller & Ulvskov, 2010). Lignocellulose is found in the primary and secondary cell walls of terrestrial plants. The three main polymers, cellulose, hemicellulose, and lignin, make up 90% of the dry matter in lignocellulosic biomass with the remaining fraction consisting of minerals, oils, and other components (Balat, 2011). The relative abundance of these polymers will vary depending on the biomass. A comparison of the relative amounts in common 2G lignocellulosic feedstocks is outlined in Table 2.

Table 2 – Typical relative abundance of the main polymers found in different types of lignocellulosic biomass. Adapted from Azadi et al. (2013).

Feedstock	Cellulose	Hemicellulose	Lignin
Softwood	40-44%	20-32%	25-35%
Hardwood	40-44%	15-35%	18-25%
Wheat straw	38%	29%	15%
Corn stover	38%	26%	19%

The three polymers are closely associated by covalent cross-linking and are responsible for the high recalcitrance of lignocellulosic biomass (Figure 3).

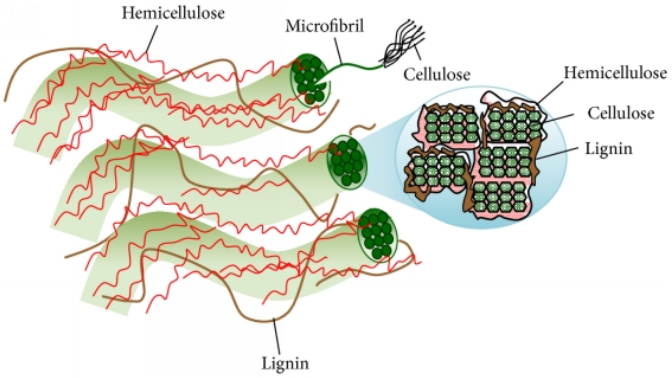

Figure 3 – Association of the three main lignocellulose polymers in the plant cell wall. Picture from Lee et al. (2014).

1.3.1 Cellulose

Cellulose is the most abundant macromolecule on earth. It is a component of lignocellulosic biomass but can also be produced by some bacterial strains (Zhong, 2020). C1 and C4 hydroxyl groups on adjacent D-glucose units are connected via β-1,4-linkages to form a linear homopolymer (Figure 4). Every other D-glucose molecule is rotated by 180° around the glycosidic bond, and the resulting cellobiose dimer is the repeating unit in the polymer (Cocinero et al., 2009). Cellulose polymers found in plant cell walls tend to be shorter than cellulose from algal (Deniaud-Bouët et al., 2014) and animal (tunicates) sources (Dunlop et al., 2018). The cellulose polymer is insoluble in water, but oligosaccharides with less than 8 D-glucose units are water-soluble (Brown Jr., 2004).

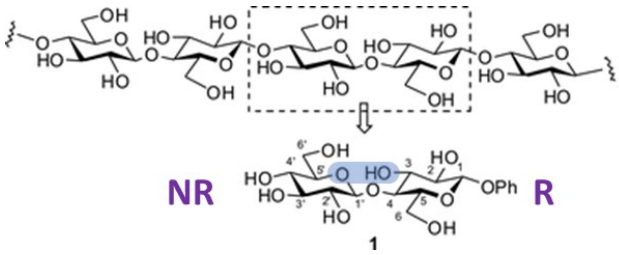

Figure 4 – Structure of cellulose and the repeating unit of cellobiose (1). Blue line in cellobiose (1) illustrates formation of hydrogen bond between OH3 and O5'. Non-reducing (NR) and reducing (R) ends are indicated for the cellobiose dimer. Figure adapted from Cocinero et al. (2009).

Cellulose polymers pack together in parallel with hydrogen bonds to form crystalline microfibrils (~3.5 nm wide), which are further aggregated into sheets (Song et al., 2020) as illustrated in Figure 3. There are two naturally occurring cellulose crystal forms: cellulose I_α and I_β. Cellulose found in the cell wall of higher plants is mainly found in the I_β form. The recalcitrant nature of the cellulose crystal is due to the network of intra- and intermolecular hydrogen bonds (H-bonds) (Shen & Gnanakaran, 2009). Besides H-bonds, van der Waals forces from stacking interactions contribute significantly to the structural stability of cellulose microfibrils (Li et al., 2011; Parthasarathi et al., 2011). Cellulose chains located on the edges of the microfibril will be less stable due to reduced H-bonds and stacking interactions from neighboring chains (Shen & Gnanakaran, 2009).

In cellulose I_β, a large number of nonpolar regions are exposed, which along with stacking interactions contributes to the hydrophobic nature of the surface of the microfibril (Parthasarathi et al., 2011). This hydrophobicity enhances binding of carbohydrate-binding domains (CBMs) to cellulose through interactions with the aromatic residues in the planar surface of the CBM (Linder et al., 1995). Treating cellulose I_β with liquid ammonia converts the structure to cellulose III_I, which is a more reactive polymer showing enhanced hydrolysis rates (Parthasarathi et al., 2011). This enhanced rate of hydrolysis is due to improved accessibility of the hydroxyl groups and glycosidic oxygens in cellulose III_I as a result of increased lattice spacing between the cellulose chains after recrystallization.

Naturally occurring cellulose microfibrils contain crystalline and amorphous regions, the relative amounts of which determine the degree of crystallinity. Degree of crystallinity varies between cellulose sources; and polymers with a high crystallinity index are, in general, more resistant to enzymatic depolymerization (Bubner et al., 2013). Kinks in the microfibril, micropores, and capillaries all contribute to the variety of cellulose sources found in nature (Lynd et al., 2002). In commercially available cellulose like the microcrystalline cellulose Avicel, amorphous regions are removed extensively by dilute acid pretreatment (Lynd et al., 2002).

1.3.2 Hemicelluloses

Hemicelluloses are heterogeneous polysaccharides that contribute to the structural integrity of the primary and secondary plant cell wall due to the close association with cellulose microfibrils (Scheller & Ulvskov, 2010). They are made up of the hexoses D-mannose, D-glucose, and D-galactose, pentoses D-xylose and L-arabinose, as well as D-glucuronic acid (Malgas et al., 2019; Moreira & Filho, 2008). The backbone of the polymer is made up of one or two of these monomers linked by β-1,4 glycosidic bonds (except in β-glucans where β-1,3 glycosidic bonds also occur) and are named after the main constituent(s) of the backbone (Figure 5). Xyloglucan, xylan, mannan, glucomannan and (mixed-linkage) β-glucan are all examples of hemicelluloses commonly found in terrestrial plants. The composition of hemicellulose varies between plant species. Xylan and xyloglucans are found in hardwoods and grasses while glucomannan is prevalent in softwoods (Scheller & Ulvskov, 2010). Substitutions and sidechains of the main polymer backbone are linked via the hydroxyl groups at the C2, C3 or C6 positions.

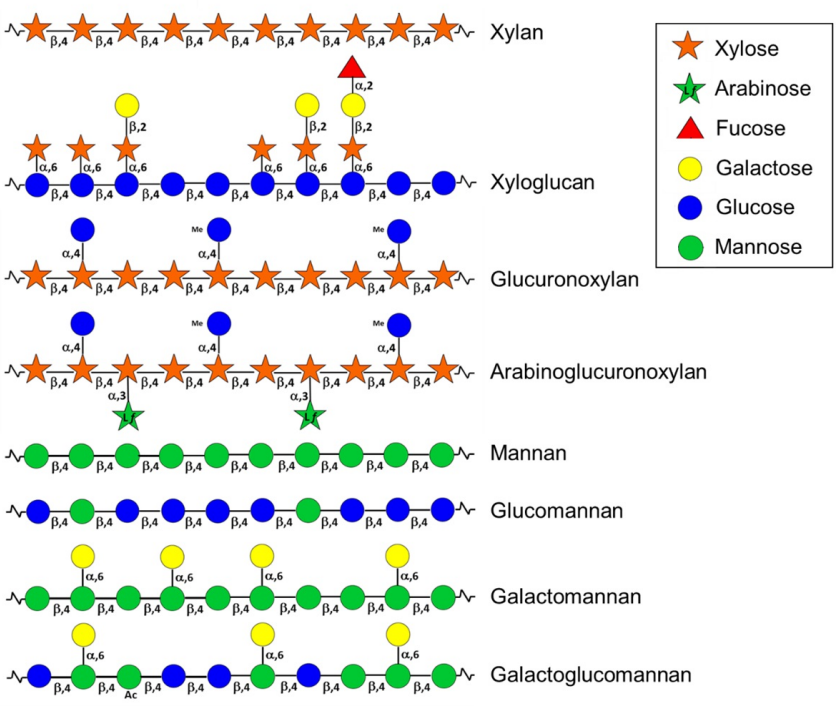

Figure 5 – Common hemicellulose structures found in terrestrial plant cell walls. Adapted from Glycopedia (Joseleau & Perez, 2022).

Xylan is made up of a backbone of β-1,4-linked xylose units which are often substituted by D-glucuronosyl and 4-*O*-methyl-D-glucuronosyl residues (Scheller & Ulvskov, 2010). Homoxylans have linear backbones with heteroxylans containing branching sugars (Malgas et al., 2019). Arabinoxylan contains L-arabinofuranose as the branching sugar at the C3 position of the backbone D-xylose residues. In glucuronoxylans, D-glucuronic acid is linked to the C2 position of the backbone D-xylose residues. Arabinoglucuronoxylans contain both branching sugars (Malgas et al., 2019). Most xylans are acetylated at the O3 position of the D-xylose residues.

Mannan backbones can be either repeating β-1,4-linked mannose residues (mannan) or a nonrepeating pattern of β-1,4-linked glucose and mannose resides (glucomannan) that can be substituted by α-1,6-linked D-galactosyl units (galactoglucomannan). Glucomannan chains are strongly adsorbed to the surface of the cellulose microfibril (Moreira & Filho, 2008). Mannans, glucomannans and galactoglucomannans are often acetylated at the C2 and C3 position of the D-mannose residue (Lundqvist et al., 2002; Scheller & Ulvskov, 2010). Galactoglucomannan is the main group of hemicelluloses found in softwoods like Norway spruce, primarily in the secondary cell wall, but arabinoglucuronoxylans are also present in small amounts (7-8% of dry weight) (Lundqvist et al., 2002; Moreira & Filho, 2008). The galactosyl substitutions of the backbone make up the hydrophilic part of the polymer and increasing galactosyl substitutions leads to increased solubility in water. Galactose side chains also prevent formation of intermolecular H-bonds, but do lead to increased intramolecular stability via intramolecular H-bonds (Moreira & Filho, 2008).

1.3.3 Lignin

Lignin is the third structural component of the plant cell wall, present at increasing concentrations in the outer layers of the plant cell wall. The complex aromatic polymer is made up of *p*-hydroxyphenyl, guaiacyl, and syringyl units, referred to as H-, G-, and S-units (Figure 6). These constituents are derived from their respective hydroxycinnamyl alcohols: *p*-coumaryl, coniferyl, and sinapyl alcohol. Additionally, other aromatic monomers (11 classes discovered to date) have been found to be naturally occurring in lignin (Vanholme et al., 2019). All the lignin building blocks originate from the general phenylpropanoid pathway starting from phenylalanine. The

monomers are linked by C–O–C and C–C interunit linkages. The majority of the linkages in native lignin are β-O-4 ether linkages (Azadi et al., 2013).

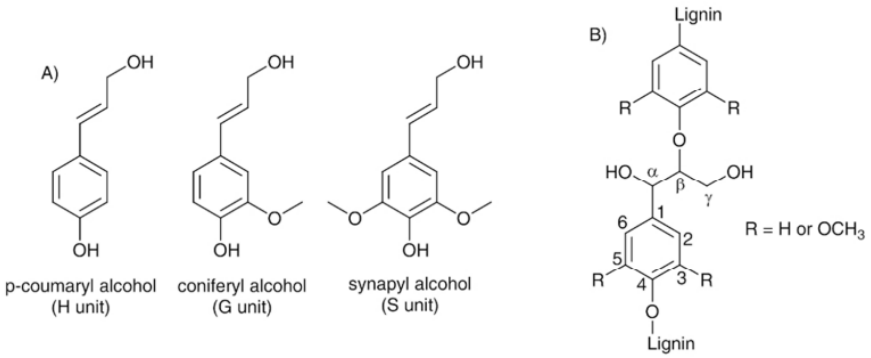

Figure 6 – The aromatic monomers found in lignin (A) and β-O-4 linkages (B) in the polymer. Figure from Fache et al. (2016).

The amount, composition, and linkages of lignin varies in different plant species, cell types, and layers of the cell wall (Vanholme et al., 2019). Softwood lignin contains 80-90% G-units and this high proportion of G-units relative to S-units results in a more recalcitrant lignin after pretreatment due to increased condensation reactions. Hardwood lignin is made up of 25-50% G-units and 50-70% S-units. Functional groups in lignin include methoxy, phenolic, alcoholic and carbonyl groups (Azadi et al., 2013).

Lignin is covalently crosslinked to hemicellulose in the plant cell wall, which leads to lignin carbohydrate complexes (LCC). This crosslink contributes to the recalcitrant nature of the plant cell wall. In grass xylan, ferulate esters have been found to be linked to the O5 position of arabinofuranosyl residues (Scheller & Ulvskov, 2010). Various ferulate dimers have been detected in grass cell walls, indicating the formation of intra- and intermolecular linkages in glucuronoarabinoxylan molecules. Ferulate esters can also form crosslinks with lignin via ether bonds (Scalbert et al., 1985). Crosslinking also takes place between lignin and glucuronoxylan via ester bonds between 4-*O*-methyl-D-glucuronic acid substitutions and aliphatic alcohol residues in lignin (Li & Helm, 1995). Additional ether linkages are formed directly between the hydroxyl groups of hexose and pentose residues in the hemicellulose backbone and sidechains and hydroxyl groups in lignin.

Valorizing the lignin fraction, which is currently underutilized, in a lignocellulosic biorefinery has the potential to greatly enhance process profitability. Lignin is the most abundant renewable source of aromatics on Earth (Azadi et al., 2013), but is resistant to physicochemical and biochemical treatment (Kamimura et al., 2019). For biochemical conversion, the high structural variation and recalcitrance makes the depolymerization of lignin into aromatic monomers the limiting step for utilizing lignin for production of bio-based products (Singhvi & Kim, 2021). Modification of lignin has been explored as a possible solution to overcome the high recalcitrance of lignin and produce a lignin that interferes less with saccharification.

1.4 Pre-treatment

Enzymatic conversion of cellulose and hemicellulose to fermentable sugars is an essential part of the biorefinery concept. The native lignocellulose feedstock is, however, very resistant to enzymatic treatment. To overcome the recalcitrance of the substrate, efficient pretreatment is an essential first step. An ideal pretreatment should result in: high recovery of carbohydrates, a digestible cellulose fraction, limited formation of degradation products, and high final concentration of solids (Galbe & Zacchi, 2007). Suitable pretreatment will vary depending on the feedstock and downstream processing steps. If for instance increased yield of fermentable sugars from saccharification is the main objective, the goal of the pretreatment is to remove lignin and separate hemicellulose and cellulose. The activity of enzymes in the saccharification step benefits from increased accessibility and surface area, reduced crystallinity and degree of polymerization, removal of lignin and hemicellulose, and other morphological changes like increased pore size (Bubner et al., 2013). An effective pretreatment should therefore aim to address these features.

Pretreatment processes can be divided into physical, chemical, physico-chemical and biological methods (Galbe & Zacchi, 2007). They can also be categorized by the impact they have on lignin. Delignification of the substrate is desirable since the presence of lignin in downstream saccharification processes may lead to negative impacts on the enzymes due to non-productive binding and blocking access to the cellulose microfibril. Kraft- and sulfite-pulping along with organosolv pretreatment solubilize and remove lignin. In contrast to this, dilute acid hydrolysis results in the partial solubilization of hemicellulose while the lignin remains in the final solid residue

with cellulose. Depending on the pretreatment, the structure of the lignin will be altered from its native state. In kraft- and sulfite-pulping processes, sulfur is incorporated into the lignin. During steam explosion, the lignin polymer is initially depolymerized into highly reactive lignin fragments that, subsequently, randomly repolymerize, resulting in a condensed lignin. This condensed lignin is more recalcitrant than its native form and negatively impacts enzyme cocktails in downstream processes (Figure 7) (Li et al., 2007; Pielhop et al., 2015). This is particularly pronounced in softwoods like Norway spruce because of the high proportion of G-units in the lignin, which undergo repolymerization reactions more readily.

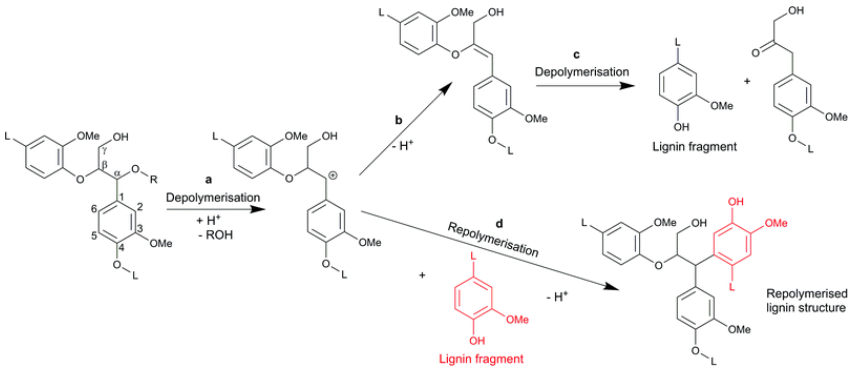

Figure 7 – An overview of lignin depolymerization (a, b, c) and repolymerization (d). Figure from Pielhop et al. (2015).

Lignin repolymerization during steam explosion can be minimized by the addition of carbocation scavengers, which are aromatic compounds that bind competitively to the carbocation preventing further repolymerization (Pielhop et al., 2016; Wayman & Lora, 1978). One study found that addition of the carbocation scavenger 2-naphthol during steam explosion of spruce could improve cellulose digestibility by 192% (Pielhop et al., 2017).

Pretreatments can be carried out under low (in the presence of an acid catalyst), neutral (no added catalyst), or high (in the presence of a base catalyst) pH conditions (Galbe & Zacchi, 2007). Steam explosion has been proven to be a suitable treatment for recalcitrant softwood feedstocks like Norway spruce. Steam explosion is carried out close to neutral pH, but due to the formation of acetic acid generated from released acetyl groups from the hemicellulose, the pH is lowered during the pretreatment. This autohydrolysis process leads to solubilization of the hemicellulose fraction, and subsequent enzymatic saccharification will require addition of hemicellulolytic

enzymes that act on the soluble oligomers to obtain a high final yield of monomeric sugars (Galbe & Zacchi, 2007). Steam explosion is often supplemented by addition of an inorganic acid catalyst like H_2SO_4 to increase conversion.

Hydrothermal pretreatments, such as steam explosion, are often compared based on their severity factor. The severity factor is calculated based on process temperature and residence time (Equation 1).

Equation 1 – Severity factor, $\log(R_0)$. t = process time (min), T = process temperature (°C), T_{ref} = 100°C

$$\log(R_O) = \left(t \times e^{\left(\frac{T - T_{ref}}{14.75}\right)} \right)$$

High severity pretreatment is required to increase accessibility and reduce crystallinity of cellulose in recalcitrant feedstocks like softwood. However, a high severity also leads to increased formation of degradation products which inhibit downstream enzymatic saccharification and fermentation processes. The degradation products are a result of hemicellulose degradation. Monomeric pentoses and hexoses are further broken down into furfural and 5-hydroxymethylfurfural (5-HMF), respectively (Figure 8). Furfural and 5-HMF can be further degraded and converted into carbon-enriched aromatic intermediates, which polymerize and form pseudo-lignin (Figure 8) (Shinde et al., 2018). Like lignin, pseudo-lignin inhibits downstream saccharification due to unproductive adsorption of the enzymes and shielding of the cellulose microfibril. Pseudo-lignin formation is increased at high pretreatment severities since this is a product of polysaccharide degradation. These effects must be balanced with the positive outcomes of the pretreatment when optimizing the process (Galbe & Zacchi, 2007). A possible solution is to divide the pretreatment into two steps, with the initial step being carried out under mild conditions that solubilize the hemicellulose fraction without formation of degradation products. After separation of this fraction, a higher severity pretreatment could be carried out to yield a less crystalline cellulose fraction. This will, however, add complexity to the process and may therefore not be a feasible solution.

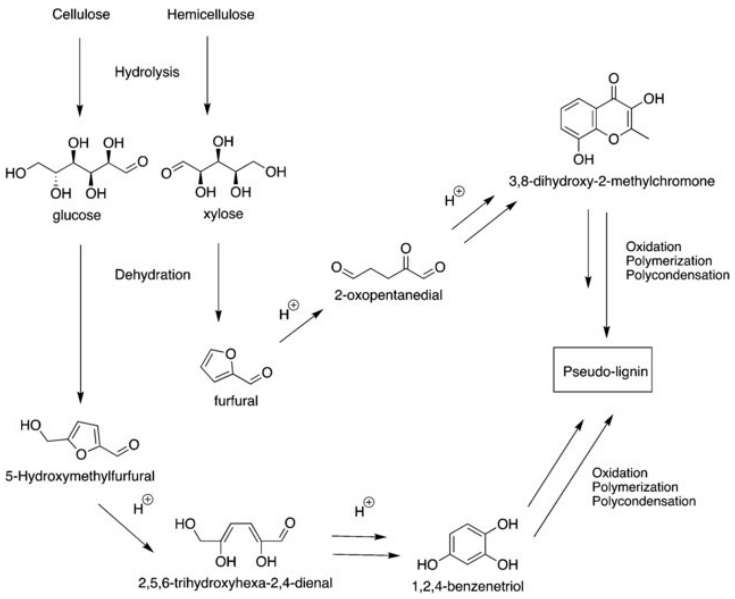

Figure 8 – Reaction pathway showing formation of degradation products 5-HMF and furfural from cellulose and hemicellulose. These compounds can further degrade and repolymerize to yield pseudo lignin. Figure from Shinde et al. (2018).

The pretreatment can be optimized based on a variety of parameters, and the optimal condition will depend on the overall process, including feedstock type and process configuration. Commonly, efficiency of enzymatic saccharification and fermentation is used to evaluate and compare pretreatments. This will elucidate effects of inhibitors on enzymes and fermenting strains. Additionally, dry matter concentration after pretreatment and formation of inhibitors will affect enzymatic saccharification and fermentation processes (Galbe & Zacchi, 2007). The interconnectedness of process steps further highlights the need for a combined process-oriented approach when optimizing the individual steps in a biorefinery.

1.5 Saccharification

Biological degradation of a recalcitrant and complex material like lignocellulose requires the combined action of a range of enzymes. Several fungal and bacterial strains, like *Trichoderma reesei* and *Clostridium thermocellum*, express a consortium of lignocellulolytic enzymes to facilitate the depolymerization of plant cell wall (Bubner et al., 2013). There are a multitude of synergistic effects between lignocellulolytic enzymes as shown in Figure 9 which lead to efficient and complete saccharification of plant

polysaccharides. Leveraging the activities of individual enzymes and synergism between them is extremely important for maximizing saccharification efficiency in a biorefinery. To achieve high conversion, and most of all economic feasibility, enzymes for industrial applications must have high stability, high activity, high resistance to inhibitors, and a high degree of synergy with other enzymes present (Malgas et al., 2019). Substrate factors that influence the efficiency of enzymatic saccharification are the associations between lignin and hemicellulose, cellulose crystallinity, and available surface area. The efficiency of the enzymes is impacted by end-product inhibition, synergistic effects, and enzyme adsorption onto lignin (Mansfield et al., 1999).

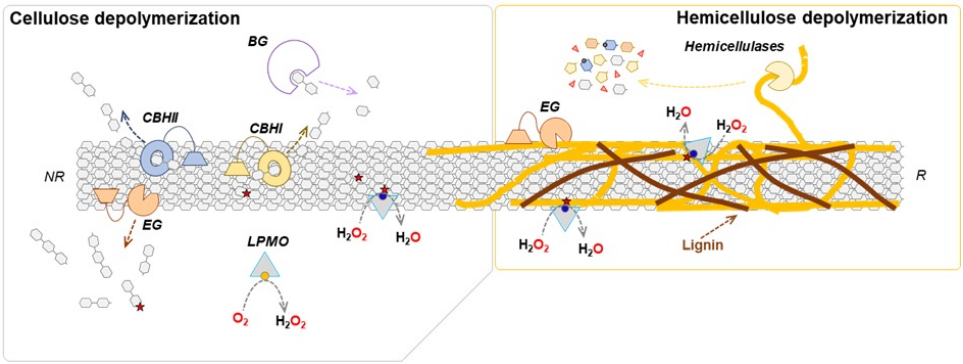

Figure 9 – The interplay of carbohydrate-active enzymes that take part in the depolymerization of the plant cell wall. CBH=cellobiohydrolase, EG=endoglucanase, BG=β-glucosidase, LPMO=lytic polysaccharide monooxygenase, NR=non-reducing end, R=reducing end. Figure adapted from Østby et al. (2020).

Cellulolytic enzymes can be linked to one or more carbohydrate-binding domains (CBMs) by a flexible linker. CBMs serve three general functions: increasing local concentration of enzymes on the surface of the cellulose microfibril (proximity effect), selectively binding the enzyme to its substrate (targeting effect), non-hydrolytic disruption of crystalline cellulose (disruptive function) (Boraston et al., 2004). The beneficial effect of CBM is more pronounced at low substrate concentrations (Várnai et al., 2013). CBMs are promiscuous proteins able to cope with the high natural variability in plant polysaccharides. Type A CBMs bind to crystalline polysaccharides, and type B CBMs bind to single polysaccharide chains (Boraston et al., 2004).

The pretreated slurry often has a high level of solids; and to produce concentrated sugar solutions, this slurry should preferably be used directly to avoid dilution steps. This poses additional challenges for the enzymatic saccharification due

to mixing limitations and a high potential concentration of inhibitors from the pretreatment (Battista & Bolzonella, 2018).

1.5.1 Cellulases

The role of hydrolytic enzymes in cellulose depolymerization has been well studied. Since cellulose is linked by glycosidic linkages, this involves the action of glycoside hydrolases (GHs) like endoglucanases (EG), cellobiohydrolases (CBH), and β-glucosidases (BG) (Figure 9) (Nidetzky et al., 1994). These act synergistically to depolymerize the cellulose microfibril. EGs cleave glycosidic bonds predominantly in amorphous regions in the center of cellulose chains (endo-activity), while CBHs act in a processive fashion on non-reducing or reducing chain ends (exo-activity), releasing cellobiose units. The activity of EGs leads to release of cello-oligosaccharides, swelling of the cellulose surface (which increases accessibility of other cellulolytic enzymes), and generation of new chain ends for the CBHs (Lee et al., 2000). Cello-oligosaccharides and cellobiose are cleaved by BGs into glucose monomers. Endoglucanases and cellobiohydrolases can be linked to one or more carbohydrate-binding modules (CBMs), which mediates binding to the surface of the cellulose microfibril as described previously. Increasing concentrations of the end products cellobiose and glucose repress activity of the cellulases and β-glucosidases, respectively (Merino & Cherry, 2007). In industrial applications, end-product inhibition will be increased due to the high total amount of sugars in solution resulting from high dry matter saccharifications.

Activity of cellulolytic enzymes is closely related to the morphology of the cellulose microfibril. The rate of saccharification is affected by adsorption, unproductive binding to the surface, site accessibility, obstacles on the surface that may negatively impact processive cellulases, and enzyme inhibition or inactivation (Bubner et al., 2013; Igarashi et al., 2011). The activity of processive celluloses is for example halted when they encounter obstacles on the cellulose microfibrils. Successive interruption of CBH activity along the microfibril leads to “traffic jams” as the CBHs do not dissociate from the polymer (Igarashi et al., 2011).

1.5.2 Hemicellulases

To improve the economic feasibility of a biorefinery, it is necessary to utilize the full potential of the lignocellulosic feedstock. If the hemicellulose fraction is not removed in the pretreatment step, this includes depolymerization of hemicellulose to fermentable hexose and pentose sugars. Hemicellulolytic enzymes also increase accessibility of the cellulose microfibril for cellulolytic enzymes (Figure 9). Depending on the severity of the pretreatment, hemicelluloses are partially broken down in this step, but an enzyme treatment is still necessary to reach full conversion. Due to the complexity of the hemicellulose structure, a variety of enzyme activities are needed for complete depolymerization.

Mannan-degrading enzyme systems have been found in bacteria and fungi and can be induced by the presence of manno-oligosaccharides and mannose monomers (Moreira & Filho, 2008). The backbone of glucomannan, the type of mannan that occur in softwood, is depolymerized by the synergistic action of endo- and exo-acting GHs in a similar fashion to the cellulolytic machinery. Glucomannan-degrading enzymes include EGs, β-mannanases, β-glucosidases, and β-mannosidases. Acetyl and galactosyl substitutions of the backbone are removed by acetylmannan esterase and α-galactosidase respectively, which increases accessibility of endo-acting enzymes (Moreira & Filho, 2008). EGs randomly cleave β-1,4 glycosidic linkages between two glucose units or between a glucose and a mannose unit. β-Mannanases randomly cleave β-1,4 glycosidic linkages between two mannose units or between a mannose and a glucose unit. β-Mannosidases and β-glucosidases cleave oligomers released by the activity of EGs and β-mannanases.

The xylan-degrading enzyme systems also contain a variety of enzyme activities and have been found in bacteria, fungi, and yeast. Some species only produce a subset of the xylan-degrading enzymes, and many produce multiple variants of the same enzyme with altered properties (Malgas et al., 2019). Synergies between β-xylanases from different GH families lead to increased xylo-oligosaccharide (XOS) production (Malgas et al., 2019). Synergistic effects are also found when combining β-xylanase and β-xylosidase, the latter of which acts on the XOS released by the endo-acting β-xylanase and are more pronounced in the simultaneous presence of these enzymes compared to their sequential addition (Malgas et al., 2019). Enzymes removing decorations and

sidechains from the xylan backbone, like α-glucuronidase and carbohydrate esterases, increase activity of endo-acting β-xylanases, which thus benefit from increased accessibility of their substrate (Malgas et al., 2019). In addition to the synergies described above, additional synergies may be found during the combined xylan depolymerization by the full consortium of secreted enzymes as discussed by Malgas et al. (2019).

As previously mentioned, lignin-carbohydrate complexes (LCCs) occur naturally in the plant cell wall, but can also form during pretreatment (Choi et al., 2007). LCCs can be cleaved by carbohydrate esterases like glucuronoyl esterases to increase activity of glycoside hydrolases (Malgas et al., 2019). A glucuronoyl esterase from the white-rot fungus *Cerrena unicolor* has been shown to significantly boost the effect of a β-xylanase during saccharification of a lignin-rich precipitate of birchwood (Mosbech et al., 2018). The enzyme cleaves the ester linkage between glucuronoxylan and lignin, releasing xylo-oligosaccharides, which are substrates for the β-xylanase.

1.5.3 LPMOs

Limited access to buried as well as highly crystalline areas of the cellulose microfibril limits the activity of cellulases. In order to increase binding sites and improve accessibility, non-hydrolytic proteins can disrupt and loosen highly organized and therefore less accessible regions of the cellulose microfibril (Arantes & Saddler, 2010). This initial step is referred to as amorphogenesis. The need for such a step was predicted in 1950 by Reese and colleagues (Reese et al., 1950). In their proposed C_1-C_x model, the enzymatic depolymerization of cellulose would progress in a consecutive two-step process. The cellulose matrix is disrupted and opened by non-hydrolytic activity (C_1 step), which allows increased accessibility of hydrolytic cellulose-depolymerizing enzymes (C_x step). As previously mentioned, hydrolytic cellulases have been well characterized but it was not until recently that enzymes and proteins contributing to the C_1 step were discovered (Arantes & Saddler, 2010; Vaaje-Kolstad et al., 2010).

Lytic polysaccharide monooxygenases (LPMOs) were recently discovered and are believed to be a key part of the C_1 step. They have since been shown to be an essential part of the cellulose-degrading enzyme system (Horn et al., 2012). LPMOs are oxidative enzymes with a flat active site that binds to the crystalline surface of the cellulose

microfibril. The active site of LPMOs consists of a highly conserved histidine brace which coordinates a single copper. This copper must be reduced for LPMOs to be catalytically active (Quinlan et al., 2011).

Initially, it was widely accepted that molecular oxygen was required for LPMO activity. However, it is now clear that the LPMO reaction is driven by H_2O_2 (Bissaro et al., 2017). H_2O_2 can be either supplied directly or produced *in situ* by reactions between O_2 and a reductant. These modes of action are referred to as the monooxygenase (using O_2) and peroxygenase (using H_2O_2) reactions (Figure 10). In the monooxygenase reaction, reductant is required throughout the duration of the reaction as opposed to the peroxygenase reaction, which only requires priming amounts of reductant. External sources of reductant like ascorbic acid and gallic acid have been used to drive LPMO activity. However, in natural systems phenolic compounds from lignin can reduce the LPMO. The active site copper in LPMOs can also be reduced directly by the action of cellobiose dehydrogenase, which can be added to the system (Tan et al., 2015). It is noteworthy that the H_2O_2-driven reaction has been shown to be 1000 times faster than the reaction with O_2 (Jones et al., 2020). It is plausible that (apparent) monooxygenase activity may be perceived when H_2O_2 is produced from O_2 *in situ* during typical LPMO reaction conditions and thus is an overlooked peroxygenase reaction.

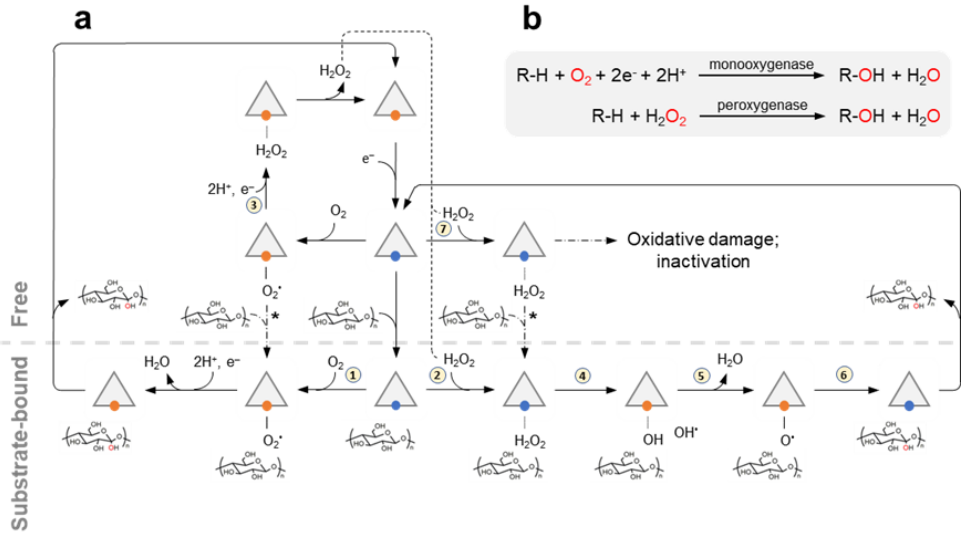

Figure 10 – Activation of LPMOs by the monooxygenase and peroxygenase reactions. Figure from Østby et al. (2020).

The co-substrate H_2O_2 can be generated by the LPMO or substrate or provided by external sources. External H_2O_2 can be produced by glucose oxidase (Manavalan et al., 2021), cellobiose dehydrogenase (Kracher et al., 2020), or supplied directly by exogenous addition (Müller et al., 2018). In the absence of substrate, free LPMOs can produce H_2O_2 but also suffer from oxidative damage, which leads to inactivation (Figure 10). This is pronounced at high local H_2O_2 concentrations (Müller et al., 2018). Reaction conditions should therefore be carefully considered to minimize LPMO inactivation.

Cellulose-active LPMOs oxidatively cleave the β-1,4-glycosidic bonds in cellulose polymer creating knicks in crystalline areas of the microfibril. The oxidative cleavage leads to oxidation at the C1 or C4 position of the reducing sugar in the cellulose chain. LPMOs are regioselective and are either C1-, C4- or C1/C4-oxidizing (Frommhagen et al., 2018). The addition of LPMOs increases activity of other enzymes in the cocktail. Access to the hydrophobic face of the cellulose microfibril has been hypothesized to be the rate-limiting step of cellulose saccharification (Ding et al., 2012). Hydrophobic binding sites are limited due to the crystalline nature of cellulose, which reduces possible binding of CBH. Strong synergy between an LPMO and cellulases was first reported by Frommhagen et al. (2015), where a 16-fold increase in cello-oligosaccharide release was achieved by the combined action of *Mt*LPMO9A and an endoglucanase. LPMO activity has also been reported for xylan and XOS (Frommhagen et al., 2015; Simmons et al., 2017). It has been suggested that xylan can only be degraded by LPMOs (AA9 or AA14) when xylan is adsorbed onto the crystalline cellulose microfibril (Couturier et al., 2018; Frommhagen et al., 2015; Tõlgo et al., 2022). It seems plausible that in nature, some AA9 LPMOs have a double function and, similar to GH7 EGs, clean cellulose microfibrils from recalcitrant xylan that strongly adheres to the surface of cellulose microfibrils (Busse-Wicher et al., 2014), as also discussed by Østby et al. (2020). Notably, synergy between LPMOs and cellulases seems to work both ways. Despite early indications, the mode of action and type of synergism between LPMOs as well as between LPMOs and cellulases needs further studying.

1.5.4 Other enzyme activities

In addition to LPMOs, other non-hydrolytic proteins from fungi and bacteria contribute to amorphogenesis such as expansins and expansin-like proteins (Arantes & Saddler, 2010). Expansins have no catalytic activity and are often connected to a CBM

by a short peptide linker. When they are in close proximity, the two domains can form a combined polysaccharide-binding surface. Synergistic effects exist between even very low levels of expansins and cellulases during crystalline cellulose depolymerization (Arantes & Saddler, 2010). Expansins disrupt the cell wall by interfering with intramolecular non-covalent bonds in the cellulose microfibril, which enhances cellulose accessibility (Arantes & Saddler, 2010). The expansin-like proteins swollenin and loosenin have been shown to disrupt crystalline cellulose substrates and enhance cellulase activity, but the exact mechanism remains unclear (Liu et al., 2015). Several swollenins are found in the commonly used cellulolytic fungal strain *T. reesei* that may work synergistically to enhance impact of the initial amorphogenesis step (Saloheimo et al., 2002).

In nature, lignin degradation is a two-step process: depolymerization followed by mineralization of the resulting aromatics. White-rot basidiomycetes like *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora* are the main lignin degraders (Kamimura et al., 2019). White-rot fungi secrete a host of oxidative enzymes including lignin peroxidases, manganese peroxidases, versatile peroxidases, dye-decolorizing peroxidases, and laccases, which depolymerize lignin by formation of phenoxy radicals (Kamimura et al., 2019; Martínez et al., 2018). The H_2O_2 needed by peroxidases is produced by oxidases like aryl-alcohol oxidase, methanol oxidase, glyoxal oxidase and unspecific peroxygenases (Kamimura et al., 2019). Possible interconnection between redox lignocellulolytic enzymes through reactive oxygen species they generate and consume in their catalytic cycle has been summarized by Bissaro et al. (2018).

Bacterial lignin degraders *Amycolatopsis*, *Streptomyces,* and *Rhodococcus* secrete dye-decolorizing peroxidase, laccase, and catalase (Kamimura et al., 2019). While fungi are mainly responsible for lignin depolymerization, lignin mineralization is carried out predominantly by bacteria. *Sphingobium* sp. SYK-6 has a well characterized aromatic lignin monomer catabolism and produces vanillate, syringate and *p*-hydroxybenzoate. Bacterial systems could be utilized to produce a variety of value-added chemicals in a biorefinery including flavor compounds like vanillin and building blocks for polymers (Kamimura et al., 2019).

1.6 Fermentation

For an efficient fermentation step in a lignocellulose biorefinery, the applied microbial strain should have a high tolerance to inhibitors originating from the pretreatment step, be able to metabolize all monomeric sugars from the substrate and be able to grow in minimal media at low pH and elevated temperatures (Zaldivar et al., 2001).

Two principal process setups are commonly studied for implementation in a biorefinery. In separate hydrolysis and fermentation (SHF), the saccharification and fermentation are carried out sequentially. In simultaneous saccharification and fermentation (SSF), they are carried out in a one-step process. Both process setups have advantages and disadvantages, and the optimum setup will depend on a variety of factors as outlined below.

The advantage of SHF is that the saccharification and fermentation can both be carried out under optimum process conditions. Lignocellulolytic enzymes have high temperature optima, while most strains grow under mesophilic conditions. Other conditions such as aeration, mixing, and nutrient requirements may also differ between the two processes. The two major disadvantages of SHF are increased cost due to a longer and more complex process setup as well as increased feedback inhibition of the enzymes by end products. The latter leads to an increased enzyme requirement and/or lower possible inclusion levels of lignocellulosic substrate since high product yields cannot be reached. The downsides to SHF are avoided in an SSF process. The advantages of SSF include reduced reactor volume due to a single reactor being used, a faster process, and reduced feedback inhibition during enzymatic saccharification. Reducing feedback inhibition leads to increased productivity of the enzymes, which enhances the rate of saccharification, lowers enzyme loading, and leads to higher product yields. SSF is often more suitable for highly accessible substrates that have been pretreated at high severity (i.e., dilute acid steam explosion) (Balat, 2011). SSF is not suitable if optimal conditions for the fermenting strain and enzyme cocktail are not compatible. In addition to the difference in temperature optima between the enzyme cocktail and the fermenting strain, aeration poses another potential compatibility challenge. Modern cellulase cocktails contain high levels of LPMOs, a key group of enzymes required for efficient saccharification. As has been previously discussed, LPMOs rely on H_2O_2

produced in the presence of molecular oxygen or supplied directly under anoxic conditions. When the LPMO reaction is driven by oxygen, SHF is superior to SSF due to competition for oxygen between the enzymes and the fermenting strain (Cannella & Jørgensen, 2014; Müller et al., 2017). Depending on the cellular metabolism, aeration may also lead to reduced yields due to side-reactions. This has up until recently been a bottleneck in designing efficient SSF processes. However, the new finding that LPMOs are activated by H_2O_2 may open new approaches to SSF processes under anoxic conditions.

Finally, the process can be integrated further by combing enzyme production, saccharification and fermentation into a single step. This is referred to as consolidated bioprocessing (CBP) or direct microbial conversion. The advantages of CBP are low capital investment and operating costs. Disadvantages are low yields and low tolerance to ethanol by the cellulase producing strain (Teter et al., 2014). These limitations could possibly be overcome by metabolically engineered strains. Although there have been successful developments towards improving CBP, like engineering expression of cellulolytic enzymes into ethanol producing yeast *S. cerevisiae* (Hasunuma & Kondo, 2012), the yields are still not sufficient for a commercial biorefinery (Maleki et al., 2021).

The ethanol producing yeast strain *S. cerevisiae* is commonly used for commercial bioethanol production. It is a robust strain with a high stress tolerance that can be genetically engineered to metabolize both hexoses and pentoses resulting from the saccharification step (Jacobus et al., 2021), making it a suitable candidate for use in a lignocellulose biorefinery. Beyond high volume products like bioethanol, value can be added to a biorefinery by production of a wide portfolio of products – especially high value chemicals. Oleaginous Zygomycete fungi have potential to be used for production of a variety of chemicals like pigments, organic acids, and biopolymers in a fermentation process utilizing lignocellulosic hydrolysates (Dzurendova et al., 2022). These oleaginous fungi can also be used for single cell oil production while single cell protein production has been demonstrated in yeast using a lignocellulosic carbon source (Lapeña et al., 2020).

1.6.1 Lactic acid production

PLA is produced from lactic acid (Lim et al., 2008), which can be produced biologically by fermentation or by chemical synthesis. The biological process has several advantages: strains that produce lactic acid with high optical purity can be selected (unlike the chemical synthesis which produces a racemic mixture) (Wee et al., 2006) and carbon sources for the fermentation can be produced from renewable sources like lignocellulosic biomass (Raj et al., 2022). Optically pure lactic acid is important for downstream polymerization reactions and for other applications of lactic acid in food, textile, cosmetics, bio-medical and chemical industries (Fan et al., 2009; Wee et al., 2006). Additional advantages include low production temperatures compared to chemical synthesis, which lowers energy consumption.

Bacterial fermentations are responsible for 90% of lactic acid produced globally with the remaining 10% produced by chemical synthesis involving acid hydrolysis of lactonitrile (Hofvendahl & Hahn-Hägerdal, 2000). For biological production of lactic acid by fermentation, the cost of feedstock (i.e. carbon source) makes up a significant proportion of production costs (Abdel-Rahman et al., 2011a). Lignocellulosic biomass is a low cost, widely available and sustainable feedstock, and an attractive candidate to replace traditionally used feedstocks like starch and refined sugars, due to the high polysaccharide content.

Production of lactic acid from lignocellulosic biomass has been demonstrated for a variety of substrates, with fed-batch setups producing the highest yields due to reduced substrate inhibition of the strain (Abdel-Rahman et al., 2011a). As previously outlined, SSF has many potential advantages for biorefinery applications. SSF of a variety of lignocellulosic feedstocks has been proven to be an effective strategy for obtaining high lactic acid productivity and yield (Pontes et al., 2021). Continuous removal of lactic acid from the fermentation medium in SSF by electrodialysis has been shown to improve lactic acid yields (Li et al., 2004), revealing potential negative impacts of elevated concentrations of lactic acid on the enzymes and fermenting strain. These detrimental effects are mostly offset by the reduced feedback inhibition of cellulases (Abdel-Rahman et al., 2011a; Iyer & Lee, 1999). Incompatible process conditions between the strain and enzyme cocktail will, however, make SSF less favorable as seen in a study by Parajó et al. (1997), where the highest lactic acid yields were reached when

performing a pre-saccharification step at higher temperature before SSF due to the higher temperature optimum of the enzyme cocktail than that of the fermentative strain.

Bacteria, cyanobacteria, yeast, fungi, and algae all produce lactic acid (Singhvi et al., 2019) by hetero- or homo-fermentation. The homofermentative process only produces lactic acid from hexoses via the Embden-Meyerhof pathway, whereas the heterofermentative process yields equimolar amounts of lactic acid, CO_2 and acetate via the pentose monophosphate pathway as shown in Figure 11 (Hofvendahl & Hahn-Hägerdal, 2000; Wee et al., 2006). Expected yields are 1:1 (g glucose: g lactic acid) for the homofermentative pathway and 1:0.5 (g glucose: g lactic acid) for the heterofermentative process. *Lactobacillus* spp. produce lactic acid by the homofermentative process and have been widely used for commercial production of lactic acid (Datta & Henry, 2006).

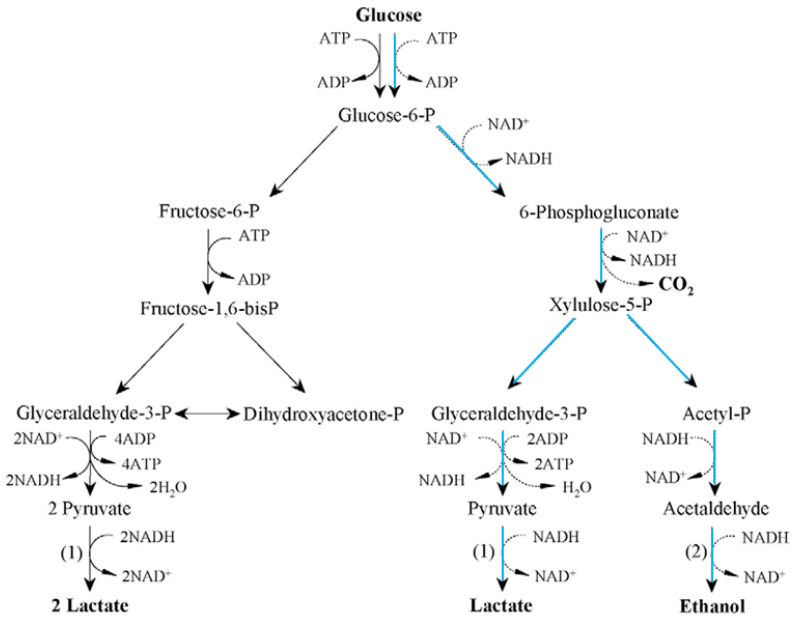

Figure 11 – The homo-fermentative (left; solid line) and hetero-fermentative (right; blue line) pathways in lactic acid bacteria. The enzymes generating the end-products are (1), lactate dehydrogenase; (2), alcohol dehydrogenase. Figure adapted from Wee et al. (2006).

Obtaining fermentable sugars from lignocellulosic biomass adds complexity to the process due to the need for costly pretreatment and enzymatic saccharification steps, which may increase the overall process costs excessively, especially for production of low-cost, high-volume chemicals like lactic acid and ethanol. All sugars should be metabolized to alleviate these costs, which causes several challenges in lactic acid bacteria (LAB) fermentations. Firstly, while hexoses are easily fermented by LAB strains, pentoses are only metabolized by a minority of LAB strains (Hofvendahl & Hahn-Hägerdal, 2000). Pentose metabolism is carried out by the phosphoketolase pathway – a heterofermentative process which will result in formation of acetic acid in addition to lactic acid (Oshiro et al., 2009; Patel et al., 2006; Tanaka et al., 2002). This not only lowers overall lactic acid yield, but also increases purification costs (Patel et al., 2006). LAB strains have been isolated or engineered to reduce formation of acetic acid and instead utilize the pentose phosphate pathway to metabolize pentose monomers (Abdel-Rahman et al., 2011b; Okano et al., 2009). Secondly, hexoses and pentoses are consumed sequentially in many bacteria due to repression of metabolism of other sugars by the presence of preferred sugars (carbon catabolite repression), which would lower overall lactic acid yield (Görke & Stülke, 2008; Stülke & Hillen, 1999). Negative effects of carbon catabolite repression can be alleviated by mixed culture fermentations (Cui et al., 2011; Taniguchi et al., 2004) or by utilizing LAB strains that can simultaneously metabolize xylose and glucose (Abdel-Rahman et al., 2011b; Guo et al., 2010). *Bacillus* spp. can utilize pentoses and hexoses (Zhou et al., 2013) to produce optically pure L-lactic acid and therefore make an attractive candidate for fermentation of complex lignocellulosic hydrolysates (Maas et al., 2008; Martinez et al., 2013). Another thing that should be considered when lignocellulosic substrates are used for production of lactic acid is the production of degradation products like furfural resulting from the pretreatment method, which are inhibitory to LAB strains. Increased resistance to furfural was achieved by addition of low concentrations of furfural to precultures in lactic acid-producing *Bacillus coagulans* DSM2314 (van der Pol et al., 2016a; van der Pol et al., 2016b).

Lactic acid yields could potentially be enhanced further by metabolically engineered strains utilizing different carbon sources like CO_2 (Upadhyaya et al., 2014; Varman et al., 2013). Strains with higher acid tolerance increase fermentation yields and reduce the amount of neutralizer needed during fermentation (Singhvi et al., 2018).

Even after simple and sustainable production of lactic acid has been realized, the polymerization reaction to form PLA requires high energy consumption (Singhvi et al., 2019). A novel direct PLA production in genetically engineered *E. coli* strains from glucose has been demonstrated (Jung & Lee, 2011; Yang et al., 2010). This one step process, however, still needs to be tested and optimized for lignocellulosic substrates.

2 Outline and purpose of the thesis

An integrated approach to not only optimize the pretreatment, saccharification, and fermentation process steps individually but also look at interconnections between these steps is key to improve product yields and profitability in a lignocellulosic biorefinery. Recent advancements in enzyme technology have revealed that inclusion of lytic polysaccharide monooxygenases (LPMOs) in a cellulase cocktail may greatly enhance saccharification yields. It has recently been shown that the action of LPMOs can be driven by addition of H_2O_2. This poses new possibilities for re-designing process setups to enable and promote LPMO activity and, consequently, boost overall saccharification yields. Accordingly, the overall aim of this thesis is to demonstrate improvement of the conversion of lignocellulosic biomass by enhancing the impact of LPMOs in saccharification and fermentation processes by modifying process conditions in the individual process steps. To achieve this goal, we have set out to pursue the following aims:

1. Summarize the recent advancement and knowledge gaps in enzymatic processing of lignocellulosic biomass, with focus on softwood as feedstock and the role of LPMOs in modern cellulase cocktails;
2. Assess the impact of pretreatment on lignin reactivity and efficiency of LPMOs in cellulase cocktails;
3. Assess if feeding H_2O_2 to enable LPMO activity is compatible with fermentation under anaerobic conditions.

This thesis is based on one review paper and two research papers:

The aim of **Paper 1** was to summarize recent developments within enzymatic processing of lignocellulosic biomass. A central topic was to show the interconnectedness between pretreatment and saccharification, with a focus on how this affects LPMOs in enzyme cocktails. Importantly, fueling LPMOs by H_2O_2, added directly or produced in situ, and the implications this has for process design in the presence or absence of lignin was elaborated.

In **Paper 2**, the aim was to investigate the effect of pretreatment on lignin reactivity and the consequence for LPMO activity in the saccharification step. The redox state of the lignin can be altered by addition of carbocation scavengers such as 2-naphthol prior to steam explosion, creating a less condensed and more reactive lignin. This work investigated the effect of 2-naphthol impregnation on LPMOs in a cellulase cocktail and the chitin-active

LPMO CBP21 (*Sm*AA10A). Moreover, pyrolysis of the lignin-rich residue was carried out and the resulting bio-oil characterized.

In **Paper 3**, direct addition of H_2O_2 to drive the action of LPMOs in a commercial enzyme cocktail during SHF and SSF processes with *B. coagulans* for production of lactic acid was examined. The feedstock was a model cellulose substrate, and H_2O_2 was added in a controlled manner by pumping it into a stirred tank bioreactor.

3 Main results and discussion

3.1 Enhancing impact of LPMOs in commercial cellulase cocktails (**Paper 1**)

This review paper provides a comprehensive overview of current developments within pretreatment and enzyme technology for lignocellulosic biomass depolymerization. For efficient saccharification to platform sugars, the pretreatment method, enzyme cocktail, and saccharification process must be developed in concert and be adapted to the starting feedstock. The main points that are highly relevant to this thesis, in particular research surrounding LPMO-facilitated lignocellulosic biomass conversion, are elaborated below.

High conversion of the lignocellulosic feedstock is essential for an economically viable biorefinery. Due to the recalcitrant nature of lignocellulosic feedstock, a pretreatment step is a critical part of this process. Novel pretreatment processes are being developed to clean off the cellulose microfibril by removing hemicellulose and lignin, to restructure native cellulose and thereby increase accessibility of enzymes, and/or to make the pretreatment process greener by repurposing biomass-derived solvents in the pretreatment step (Cantero et al., 2019; Sun et al., 2016; Yang & Wyman, 2008). Depending on the choice of pretreatment, the optimal composition of the enzyme cocktail will vary as some pretreatments alter the structure of the main polymers and some retain hemicelluloses (Banerjee et al., 2010; Chylenski et al., 2017a; Kallioinen et al., 2014). It is therefore not surprising that one of the main considerations for process optimization involves choice of feedstock and pretreatment method. Other important considerations relevant to the work in this thesis include: the choice of enzymes and optimizing separate (SHF) or simultaneous (SSF) saccharification and fermentation processes, all three considerations being notably dependent of each other.

Lignin can be either removed or retained as part of the slurry following pretreatment (Figure 12). Depending on the severity of the pretreatment, the structure and reactivity of remaining lignin may be altered. Dilute-acid pretreatment, steam explosion or hydrothermal pretreatment are all examples of lignin-retaining pretreatments where lignin can drive the LPMO reaction during the saccharification process (Harris et al., 2010; Müller et al., 2015; Westereng et al., 2015). However, the presence of lignin also causes several inhibitory effects due to nonproductive binding of the cellulases and shielding of the cellulose microfibril (Djajadi et al., 2018; Rahikainen et al., 2013). This tradeoff is a limitation to improving

efficiency of the saccharification process in lignin-rich substrates. At the Norwegian biorefinery, Borregaard, removal of lignin by a sulfite pretreatment in the 'BALI' process has proven to be suitable for production of carbohydrate and lignin streams which can be turned into valuable products (Chylenski et al., 2017b; Costa et al., 2020).

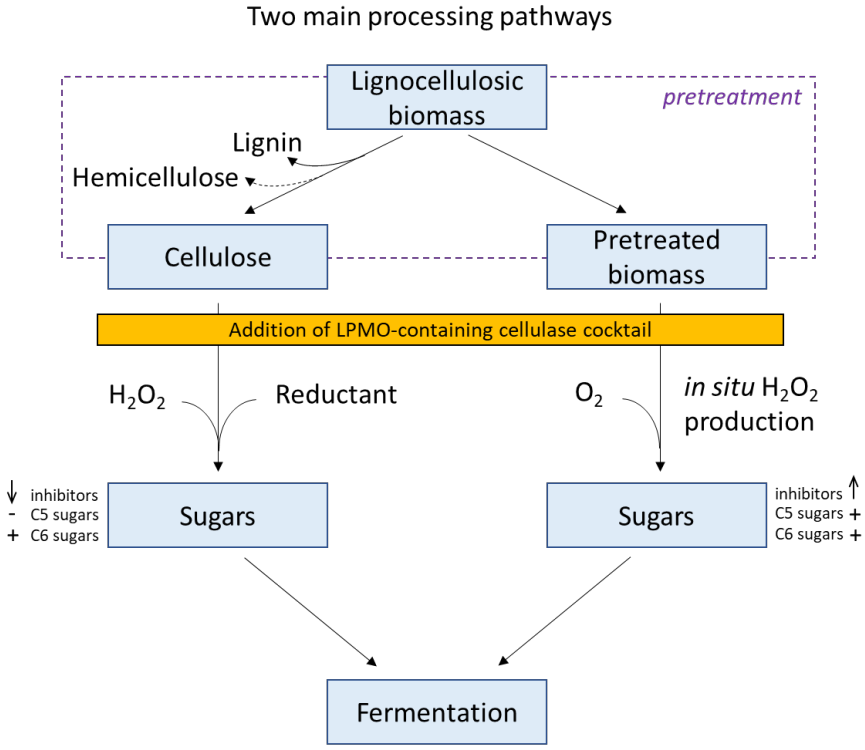

Figure 12 Schematic overview of two main processing pathways in the biochemical conversion of lignocellulosic biomass based on discussions in Paper 1. Production of platform sugars in a saccharification step can be either preceding (SHF) or combined with (SSF) a fermentation step. Lignin and hemicellulose can be separated in the pretreatment step yielding a cellulose-enriched fraction (left pathway) or remain as part of the whole slurry in further downstream processing steps (right pathway).

Due to the complexity of the substrate, it is necessary to design enzyme cocktails with a wide spectrum of activities. Hydrolytic enzymes have commonly been used in industrial processes, and the interplay between them is well studied. During the past decade, development of enzyme cocktails has expanded since oxidative cellulose depolymerization by LPMOs has gained increasing attention (Harris et al. (2014), **Paper 1**). Since their discovery, the catalytic mechanism of LPMOs, their interactions with hydrolytic enzymes, and how to harness their activity under industrial conditions have been studied extensively as reviewed by Chylenski et al. (2019) and **Paper 1**. Synergistic effects have been shown for chitin- and cellulose-active LPMOs and other hydrolytic enzymes leading to improved

substrate degradation. The LPMO reaction is driven by H_2O_2, supplied directly by an external source, or generated via abiotic reactions by lignin or oxidoreductases present in the enzyme cocktail as reviewed recently by Bissaro et al. (2018). Lignin can be treated with laccases, which further increases H_2O_2 production (Perna et al., 2020). For *in situ* production of H_2O_2, a high amount of reducing equivalent is needed as this is consumed for the duration of the reaction. In lignocellulosic substrates, lignin and phenolic compounds originating from lignin are used as the reductant. In the absence of lignin, LPMOs can be activated by direct addition of H_2O_2 and an external reductant like ascorbic acid or, in applied settings, spent sulfite liquor, which has been demonstrated in lab- and demonstration-scale (Costa et al., 2020; Müller et al., 2018), but still needs further optimization.

To fully utilize the potential of oxidoreductases for lignocellulosic biomass conversion, further research is required to elucidate lignin-interactions, flow of electrons, and the generation and consumption of H_2O_2 (**Paper 1**). The flow of H_2O_2 is impacted by lignin-active enzymes, which could be a possible link between lignin-degrading systems and cellulolytic enzymes (Bissaro et al., 2018). These two systems are further entangled since *in situ* production of H_2O_2 by LPMOs can be utilized by lignin-degrading peroxidases (Paper 1: Fig. 2).

The type of feedstock and the choice of pretreatment affects the efficiency of an enzyme cocktail. LPMO activity in the cocktail needs to be balanced keeping this in mind. Importantly, the amount of H_2O_2 generated *in situ* during the saccharification reaction may be more relevant than the amount of LPMOs in the enzyme cocktail. An increasing number of studies (Kadic et al. (2021); Müller et al. (2018), **Paper 2**) indicate that a fraction of the LPMOs in the cellulase cocktail remain inactive. This latent potential of the LPMOs in the cocktail could be realized by carefully considering (external) H_2O_2 dosing and *in situ* generation of H_2O_2 either by LPMOs or other oxidoreductases (Bissaro et al., 2018) or abiotic reactions (Stepnov et al., 2021). The overall efficiency can also be improved by optimizing existing synergies between cellulases and LPMOs in the cocktail. The type of LPMO should also be considered when designing enzyme cocktails for 2G lignocellulose biorefineries, and this is not always straightforward, as substrate-binding affinity and H_2O_2 production varies not only for feedstock and enzyme combinations but also over the course of saccharification. When relying on *in situ* generated H_2O_2 for driving the LPMO reaction, we need a careful balance between LPMOs in solution (generating H_2O_2) and LPMOs bound to the substrate (consuming H_2O_2), as illustrated by the seminal work of Stepnov et al. using an LPMO with

and without its CBM (Stepnov et al., 2022b). Despite our limited understanding of how LPMOs act in biomass processing (Chylenski et al., 2019), early works indicate that the performance of LPMO-containing cellulase cocktails could be improved by addition of selected AA9s (Merino & Cherry, 2007), highlighting the underexplored complementarity that exists between LPMOs as well as between LPMOs and cellulases. Despite extensive work on deciphering LPMO action (Eijsink et al., 2019), one of the key challenges that remains is how to control H_2O_2 levels in complex, industrially relevant systems. Excess H_2O_2 leads to LPMO inactivation, which not only lowers overall saccharification yields but also causes several undesired consequences due to release of free copper into solution (Stepnov et al., 2021a). Free copper causes a fast increase in H_2O_2 production in the presence of a reductant, and is especially pronounced for the commonly used reductant, ascorbic acid (Stepnov et al., 2022). This perpetuates further LPMO inactivation and copper release which fuels this cycle.

The inclusion of LPMOs in commercial cellulase cocktails have posed new requirements on process conditions. To activate the LPMOs by the monooxygenase reaction, reactors must be aerated to supply the enzymes with molecular oxygen. On the other hand, activation by the peroxygenase reaction requires a continuous supply of liquid H_2O_2 (if it cannot be generated *in situ*), which also poses challenges for process design. The strategy for LPMO activation also impacts the suitability of SHF and SSF processes due to requirements of the fermenting organism. In **Paper 1**, we proposed that, for lignin-poor feedstocks, driving LPMO reaction may be possible during fermentation, e.g., in an SSF setup, with a direct supply of H_2O_2 at low concentrations, which could boost saccharification and, consequently, fermentation product yields without leading to inactivation of LPMOs. Driving the LPMO reaction with feeding H_2O_2 instead of aeration would avoid competition for oxygen between LPMOs and the fermenting strain.

3.2 Upgrading pretreatment method to boost LPMO performance (**Paper 2**)

The presence and type of lignin leads to several undesired consequences in the saccharification step, as discussed in **Paper 1**. In **Paper 2**, we studied how lignin structure and reactivity affects LPMO activity. Previous work by Pielhop et al. has shown that impregnation of a softwood with a carbocation scavenger prior to steam explosion enhances subsequent enzymatic saccharification (Pielhop et al., 2017; Pielhop et al., 2016). Steam explosion is an efficient pretreatment method that leads to increased accessibility of the cellulose microfibril and solubilization of the hemicellulose fraction. During the pretreatment, lignin is depolymerized; however, due to the high amounts of radicals, lignin also quickly reassociates into a more condensed polymer. This is especially pronounced in softwoods due to the high proportion of guaiacyl residues in the lignin (Li & Gellerstedt, 2008). This condensed lignin sterically hinders access to cellulose and causes nonproductive adsorption of cellulases to a larger extent compared to native lignin (Pielhop et al., 2015). Pielhop et al. have shown that the addition of a carbocation scavenger prevents repolymerization of lignin and leads to a more efficient saccharification step, which they attributed to reduced nonproductive adsorption of cellulases onto lignin and improved cellulose accessibility by reduced recondensation of lignin that shields the cellulose polymer (Pielhop et al., 2015). Importantly, while lignin is known to play an important role in driving LPMO reaction, the effect of this less condensed lignin on LPMO activity has not been explored. Reactions between lignin and O_2 leads to formation of H_2O_2, which drives LPMO activity. Thus, in this study we wanted to investigate the effect of applying the carbocation scavenger 2-naphthol during pretreatment on subsequent LPMO activity during the saccharification step.

Batches of milled Norway spruce were impregnated with 2-naphthol overnight and pretreated in a steam explosion unit at various temperatures and residence time leading to severities between 3.64–4.53. Based on compositional analysis of the pretreated samples, it was seen that higher severity pretreatment led to increasing relative concentration of lignin, and loss of the main hemicellulose polymers. These trends were observed for both untreated and 2-naphthol-impregnated samples. Solid state nuclear magnetic resonance spectroscopy (SSNMR) analysis revealed that impregnated lignin retained more phenolic OH groups after steam explosion compared to the untreated samples. It has previously been shown that

diphenolic groups are involved in electron transfer required for the monooxygenase process for activation of LPMOs (Kracher et al., 2016).

To assess the impact of 2-naphthol impregnation on saccharification efficiency, the pretreated samples were subjected to saccharification using the LPMO containing cellulase cocktail Cellic CTec2 under aerobic conditions. Expanding on previous work by Pielhop et al. (Pielhop et al., 2017), all reactions were carried out at 10% dry matter, which is closer to industrially relevant conditions. The highest final glucose concentration could be reached with samples pretreated at 210°C and 220°C (Paper 2: Fig. S5). Based on this initial screening, the untreated and 2-naphthol-impregnated samples pretreated at 210°C and 220°C were selected for further testing with various doses of Cellic CTec2. In the subsequent saccharification experiments, 2-naphthol impregnation led to a 51-62% increase in glucan yields compared to untreated samples when the steam explosion was carried out at 220°C, even reaching 100% in one reaction (Paper 2: Fig. 1C, D). The same effect was not detected for the samples treated at 210°C, where 2-naphthol impregnation did not make a substantial difference (Paper 2: Fig. 1A, B). In control reactions with the LPMO-poor cocktail Celluclast, 2-naphthol impregnation led to a 42-51% increase in glucan yields in the samples pretreated at 220°C but did not improve yields for the 210°C samples (Paper 2: Fig. 1). We also assessed LPMO activity by quantifying the C4-oxidized dimer Glc4gemGlc, which is the main LPMO product. From these results, it is clear that 2-naphthol impregnation leads to increased formation of LPMO products in all Cellic CTec2 reactions except for the lowest enzyme dose (Paper 2: Fig. 2). LPMO activity in reactions with untreated samples was less dependent on enzyme dose compared with 2-naphthol-impregnated samples (Paper 2: Fig. 2). This means that the LPMO activity was not limited by the amount of enzyme but rather something else in these reactions. This might be availability of *in situ* generation of the co-substrate H_2O_2. If the 2-naphthol impregnation leads to a more reactive lignin capable of producing more H_2O_2, this could explain the increased LPMO activity when using this substrate. In reactions with 2-naphthol-impregnated substrate (Paper 2: Fig. 2B, D), LPMO activity is enzyme dose-dependent, and the amount of enzyme is limiting in these reactions. The enhanced saccharification of impregnated samples using Cellic CTec2 and Celluclast can be explained by decreased non-productive binding and shielding of the cellulose polymer, as also reported by Pielhop et al. earlier (Pielhop et al., 2015). These factors will impact all enzymes in the cocktail. Since the impact on glucan yield was more pronounced in the presence of LPMOs, this increase could

also possibly be explained by increased LPMO activity in addition to other beneficial effects of the 2-naphthol impregnation.

To investigate this further, we set up reactions with the chitin-active LPMO CBP21 to see if activity on β-chitin could be boosted by the lignin resulting from 2-naphthol impregnation. Since CBP21 is not active on cellulose, this allows for lignin effects that are boosting LPMO activity to be decoupled from other factors. In agreement with our previous findings, 2-naphthol impregnation led to increased CBP21 activity (Paper 2: Fig. 3). The effect was more pronounced for the sample pretreated at 220°C, but the impregnated sample pretreated at 210°C also led to higher CBP21 activity compared to its untreated equivalent. This verified our hypothesis that 2-naphthol impregnation prior to steam explosion results in a lignin that is more reactive and probably led to more production of H_2O_2, which caused the higher LPMO activity.

Building on previous work by Kalyani et al. (Kalyani et al., 2017), we were interested in exploring how 2-naphthol impregnation might influence downstream processing in a biorefinery using Norway spruce as a feedstock. In previously published work by Kalyani et al. (Kalyani et al., 2017), bio-oil produced by pyrolysis was shown to be a viable end-product. To investigate the suitability of the 2-naphthol process for this, lignin-rich saccharification residues with varying degrees of residual carbohydrates were subjected to pyrolysis. Confirming previous findings by Kalyani et al. and others (Dong et al., 2019; Kalyani et al., 2017), pyrolysis fractions resulting from lignin-rich samples contained high amounts of char (Paper 2: Fig. 4A). Even though the bio-oil fraction was reduced, it was of high quality in samples with a high degree of lignin. The bio-oil fractions were enriched in monoaromatic phenols and contained reduced amounts of sugar derived furanics and anhydrosugar products (Paper 2: Fig. 5). This increases storage stability of the oil and makes it suitable for production of biobased resins and solvents. The beneficial effects on downstream thermochemical processing steps are a result of the high degree of conversion in the saccharification step which yields a very pure lignin fraction.

These results show that 2-naphthol impregnation of Norway spruce prior to steam explosion can lead to a reduced enzyme requirement due to formation of a less condensed and hence more reactive lignin. This highlights the importance of the redox state of the lignin when LPMOs are present in modern cellulase cocktails, which needs to be considered when choosing pretreatment strategy. The fact that 100% glucan conversion of the recalcitrant substrate Norway spruce was achieved in this study underscores the large impact of the

addition of a carbocation scavenger. High saccharification yields also produce a lignin fraction with high purity that is suitable for further thermochemical processing.

3.3 Revisiting process configuration in light of new LPMO findings (**Paper 3**)

LPMOs are known to boost conversion of cellulose during enzymatic saccharification. Due to their requirement for molecular oxygen, running the saccharification independently from fermentation in a separate hydrolysis and fermentation (SHF) setup is believed to be more advantageous than a simultaneous saccharification and fermentation (SSF) process. This is due to the competition between the microbe and enzyme for molecular oxygen. But in light of the new finding that LPMOs utilize H_2O_2 as a co-substrate, this notion should be revisited. In **Paper 3**, we evaluated the feasibility of lactic acid production in SHF and SSF setups with a continuous H_2O_2 feed to activate the LPMOs.

First, we established a baseline for the saccharification reaction of Avicel in the fermenters and in the presence of media components required for cultivation of the fermenting strain. H_2O_2 was supplied directly by continuous pumping to activate LPMOs under anoxic conditions. Since Avicel is a highly crystalline cellulose substrate with no lignin, the external reductant, ascorbic acid, was added to all reactions. Cellic CTec2 was compared to the LPMO-poor cocktail Celluclast to study the activity of LPMOs under the different reaction conditions. As has been previously observed (Müller et al., 2018), a trade-off exists between H_2O_2 and final glucose concentration. Up to a certain extent, increasing levels of H_2O_2 leads to increased LPMO activity. However, too much free H_2O_2 in the system leads to LPMO inactivation and a termination of the saccharification process, as is observed for the highest feed rate tested, 160 µM/h H_2O_2 (Paper 3: Fig. 1B). A very recent study has suggested that LPMO inactivation leads to release of free copper, which will quickly react with and degrade ascorbic acid (Stepnov et al., 2022a) and also possibly degrade H_2O_2 to reactive oxygen species (Pham et al., 2013). In fact, the degradation of the Glc4gemGlc at the later phase of saccharification with 160 µM/h H_2O_2 could be indicative of a system where free copper leads to radical formation. In the control reactions, a feed rate of 80 µM/h H_2O_2 led to the highest glucose release (Paper 3: Fig. 1A). LPMO activity was established by quantifying the C4-oxidized dimer Glc4gemGlc. In all reactions except for the highest H_2O_2 feed rate (160 µM/h), LPMO product levels correlated directly to glucose release, (Paper 3: Fig. 1A, B) showing that LPMOs boost activity of the cellulases in the cocktail. It is interesting to note that the LPMO-

poor cocktail Celluclast outperformed Cellic CTec2 under anoxic conditions (Paper 3: Fig. 1A). This is likely due to the higher total amount of cellulases in Celluclast compared to Cellic CTec2, which is estimated to contain up to 20% LPMOs (Müller et al., 2015).

Bacillus coagulans was selected as the fermenting organism for SSF and SHF reactions, due to the thermophilic nature of the strain and its high lactic acid productivity. The former feature allows us to run the fermentations at 50°C, which is optimal for both enzyme and strain performance. The SSF and SHF experiments were carried out identically to the saccharification experiments, but with the addition of a *B. coagulans* inoculum at 0 and 24 hours, respectively. H_2O_2 was supplied throughout the 48-hour reaction in both cases.

Looking at glucose production, a small spike in initial glucose can be seen after 2 hours in all SSF reactions before the apparent glucose concentration reaches 0 g/L at 12 hours (Paper 3: Fig. 2B). For the SHF reactions, glucose accumulates in the first 24 hours, with all reactions with H_2O_2 pumping producing the highest final concentration of glucose (37-39 g/L). Glucose levels decrease after addition of the strain in SHF, but the growth is delayed and slower in the presence of the high levels of H_2O_2, which could indicate that the strain is suffering from oxidative stress in these reactions. This is also indicated by the accompanying slower rate of lactic acid accumulation (Paper 3: Fig. 2A, E). The concentration of the LPMO product, Glc4gemGlc, increases in the first 24 hours of the SHF setup, but declines after addition of the strain, indicating that *B. coagulans* is able to metabolize this compound (Paper 3: Fig. 2C). This could also explain why LPMO product levels were low in all SSF reactions. In all SHF reactions except for anoxic reactions with Cellic CTec2 and Celluclast, there is remaining unmetabolized glucose after 48 hours, which could be utilized by extending fermentation times (Paper 3: Table 2).

In both SHF and SSF setups, the lactic acid yields were substantially lower than what would be expected based on the saccharification experiments (assuming 1:1 conversion from glucose to lactic acid by *B. coagulans*). Under aerobic conditions, however, side product formation of acetate has been previously observed (Müller et al., 2017), which will lower overall lactic acid yields. (Acetate was not detected in our experiments). Although the LPMOs have a high affinity for H_2O_2, this discrepancy (i.e., substantially lower lactic acid yields than what would be expected from the saccharification experiments) can be explained by some of the H_2O_2 being taken up by the strain and thus lowering the LPMO activity and glucose release.

Even though LPMO activity could not be monitored in SSF reactions based on LPMO product accumulation, the lactic acid yields improved in reactions with suspected increased LPMO activity due to either the presence of O_2 or H_2O_2 (Paper 3: Fig. 2D, F). The highest lactic acid yields during SSF were obtained in the reaction setups that corresponded with the highest glucose yields in the saccharification reactions. On the other hand, the lactic acid yields after 48 hours in the SSF process were lower than would be expected based on the saccharification reactions, as discussed in the previous paragraph. The simultaneous presence of the enzymes and the strain leads to a variety of interactions that may be responsible for this lowered yield, like possible removal of H_2O_2 by bacterial catalases (Dowds, 1994; Vassilyadi & Archibald, 1985). Negative effects of H_2O_2 were less pronounced in SSF setups compared to SHF, which may be due to accumulation of high concentrations of H_2O_2 after 24 hours of saccharification, at the time when the inoculum was added. Lactic acid accumulation might also negatively impact enzyme activity (Iyer & Lee, 1999; van der Pol et al., 2016b), especially towards the end of the reaction.

Considering available Avicel in the reaction, lactic acid yields after 48 hours in the SSF process reached 24-47%. Avicel is a model substrate and difficult to degrade, so these yields will likely be higher using a more accessible substrate. Overall, the highest lactic acid yields were obtained in SSF with H_2O_2 feeding at 80 μM/h. It has previously been determined that when the LPMO reaction is driven by O_2, SHF is preferred to an SSF process. However, our results show that if the LPMO reaction is driven by direct addition of H_2O_2, it is possible to produce lactic acid by an SSF process.

4 Concluding remarks and future perspectives

The work included in this thesis highlights the critical role played by LPMOs in commercial cellulase cocktails during saccharification of lignocellulosic biomass. The positive impact of increased LPMO activity on saccharification efficiency has been previously established as we describe in **Paper 1** and is further strengthened in **Papers 2 and 3**, where we see concurrent increase in LPMO activity and glucan yields. Here, we push the limits beyond the state of the art and show that the activity of these enzymes can be further boosted and taken advantage of more efficiently, by modifying process conditions and setups.

The saccharification results in **Paper 2** and **Paper 3** strongly support previous suggestions that indicate that only a fraction of LPMOs in the cellulase cocktail is utilized during the reaction and that LPMO activity is not limited by the amount of LPMOs in the commercial cellulase cocktail Cellic CTec2, but rather by another factor. We hypothesize that this might be the *in situ* generation of H_2O_2. If only a fraction of the LPMOs present in the cocktail are active, there is a lot of unused potential in the cocktail. This is perhaps best highlighted by the finding in **Paper 2** that production of C4-oxidized dimer is not dependent on the dose of LPMOs for the saccharification of steam-exploded spruce (without 2-naphthol impregnation). The possibility to replace a fraction of inactive LPMOs with active cellulase or hemicellulase components, in general, has an overarching implication for enzyme cocktail design. Adjusting enzyme screening processes and perhaps also growth conditions during fungal enzyme production, especially in case of on-site enzyme production, has a high potential to generate more efficient enzyme cocktails.

Previous studies demonstrate that too much H_2O_2 leads to inactivation of LPMOs. In **Paper 2**, we show that 2-naphthol impregnation produces a more reactive lignin that leads to faster and prolonged product accumulation by LPMOs. If the level of externally added or *in situ* produced H_2O_2 is too high, the LPMOs are inactivated as observed in **Paper 3**. Based on recent findings, this is amplified by the release of free copper from the active site of inactive LPMOs. The complementary and synergistic effects between LPMOs remain underexplored and could be one way to reduce inactivation, by balancing the presence of high H_2O_2-producing LPMOs and LPMOs with strong binding affinity, the latter of which will consume rapidly the H_2O_2 generated by the former.

Lignin-mediated effects on LPMO activity and enzyme cocktail performance during the saccharification should be considered when pretreatment steps are optimized. In **Paper 2**, we have shown how lignin can be altered to drive LPMO reactions more efficiently. SSNMR analysis revealed that 2-naphthol impregnation led to retention of phenolic OH groups in the lignin after steam explosion. By impregnation of Norway spruce with 2-naphthol prior to steam explosion, we could increase glucan conversion up to 62% in high dry matter saccharifications (10%) and moderate enzyme dosage (16 mg/g). At the highest severity and enzyme dose, we were able to obtain unprecedented levels of conversion of the cellulose polymer (close to 100%) for steam-exploded spruce. Lignin treated with 2-naphthol boosted activity of the chitin-active LPMO CBP21 to a higher extent than untreated lignin, which is indicative of more H_2O_2 production. By using a chitin-active LPMO, we were able to exclude effects related to cellulose degradation and focus solely on the impact of lignin on the LPMO reaction. In doing so, we showed the impact of pretreatment choice on substrate recalcitrance and the overall efficiency of the cellulase cocktail. This finding redefines how the lignin content of the pretreated feedstock may be evaluated, shifting focus from quantity (i.e., lignin content) to quality (i.e., lignin reactivity). Rather than focus on delignification of the feedstock, the lignin can be modified to increase efficiency of saccharification processes separately, as shown here, or possibly also in combination with fermentation processes.

H_2O_2 pumping to drive the LPMO reaction in the absence of lignin, as discussed in **Paper 1**, will require novel process design in saccharification and fermentation processes. In **Paper 3**, we were able to show that by continuous pumping of H_2O_2 to drive LPMO reactions, we could achieve a more efficient SSF setup for lactic acid production from Avicel. Further work on industrial substrates should be tested as these often have higher accessibilities to enzymes, which may increase product yields. The presence of free H_2O_2 (at elevated concentrations) seems to negatively impact the strain in SHF reactions, which could be potentially avoided by testing various H_2O_2 addition regimes (with the aim to minimize H_2O_2 accumulation in the reaction). These results confirm that it is possible to leverage the activity of oxidative enzymes like LPMOs in SSF processes, which creates a simpler process more suited for industrial scale-up.

As mentioned in **Paper 1**, one of the main objectives in current attempts to improve pretreatment is the separation of lignin and hemicellulose from cellulose. The results in **Paper 2**, however, show that it is possible to extract value from multiple fractions in a biorefinery simultaneously as the cellulose can be degraded in an efficient saccharification step and the resulting lignin-rich saccharification residue can be used for further thermochemical conversion. In order to fully leverage the power of LPMOs in commercial enzyme cocktails, we need a better understanding of the impact of reactive oxygen species on LPMO activity and LPMO inactivation in saccharification of industrially relevant lignocellulosic substrates. To build on the results in this thesis, future work in this field should aim to 1) elucidate structural changes in the less condensed and more reactive lignin produced by 2-naphthol impregnation, 2) explore various H_2O_2 addition strategies in an SSF process, and 3) gain a deeper understanding of LPMO side reactions that may lead to inactivation, including strategies to handle release of copper from LPMOs. While there is still a lot to uncover before LPMO potential may be fully exploited, the findings of this thesis bring us closer to realizing the full potential of a softwood biorefinery.

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6 Publications

Paper 1

Enzymatic processing of lignocellulosic biomass: principles, recent advances and perspectives

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BIOENERGY/BIOFUELS/BIOCHEMICALS - REVIEW

Enzymatic processing of lignocellulosic biomass: principles, recent advances and perspectives

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Abstract
Efficient saccharification of lignocellulosic biomass requires concerted development of a pretreatment method, an enzyme cocktail and an enzymatic process, all of which are adapted to the feedstock. Recent years have shown great progress in most aspects of the overall process. In particular, increased insights into the contributions of a wide variety of cellulolytic and hemicellulolytic enzymes have improved the enzymatic processing step and brought down costs. Here, we review major pretreatment technologies and different enzyme process setups and present an in-depth discussion of the various enzyme types that are currently in use. We pay ample attention to the role of the recently discovered lytic polysaccharide monooxygenases (LPMOs), which have led to renewed interest in the role of redox enzyme systems in lignocellulose processing. Better understanding of the interplay between the various enzyme types, as they may occur in a commercial enzyme cocktail, is likely key to further process improvements.

Keywords Lignocellulose · Saccharification · Cellulase · Hemicellulose · Lytic polysaccharide monooxygenase

Introduction

Industrial-scale production of cellulosic ethanol based on enzymatic saccharification of biomass was established by several companies during the past decade [17, 298]. This production of cellulosic ethanol was initiated in 2012 by Beta Renewables at their site in Crescentino, Italy [55]. In 2015, this plant had an annual production of about 40,000 tons of ethanol using agricultural residues as feedstock. In 2017, however, this plant was shut down due to economic problems in the parent company Mossi Ghisolfi Group and sold to Versalis [107]. In early 2020, Eni, an integrated energy company owning Versalis, announced that bioethanol production in Crescentino will start again within the first half of 2020 [98]. Other companies like DuPont, Abengoa and GranBio have all had commercial plants in operation, but they have closed down production of ethanol due to economic and/or technical reasons. The POET-DSM Advanced Biofuels, a 50/50 joint venture between Royal DSM (Netherlands) and POET LLC (USA) demonstrated stable industrial production of bioethanol. Their Project Liberty facility in Emmetsburg, Iowa (USA) produced for some time around 80 million liters of ethanol per year and had an 80% uptime in 2017. However, also POET-DSM has now paused ethanol production at the site due to challenges with implementing the recent Renewable Fuel Standard [277]. Thus, the establishment of this industry has clearly been challenging, and it is currently also struggling with a low oil price.

Conversion of lignocellulosic biomass to ethanol involves five main steps, namely collection and delivery of feedstock to the plant, pretreatment of the feedstock (at the point of collection or on-site), enzymatic saccharification, fermentation and product formulation (see Fig. 1). In order to make the process viable, all these steps need to be considered from the economic point of view, with primary focus on feedstock handling, pretreatment and enzyme efficiency and enzyme costs [4, 383]. In this review, we will give an overview of recent technical improvements regarding pretreatment technologies that have been used at (semi-)industrial scale and then discuss in detail challenges and recent advancements regarding enzyme cocktails used for saccharification of lignocellulosic biomass. We will focus on enzyme components that are critical for maximizing sugar recovery from the

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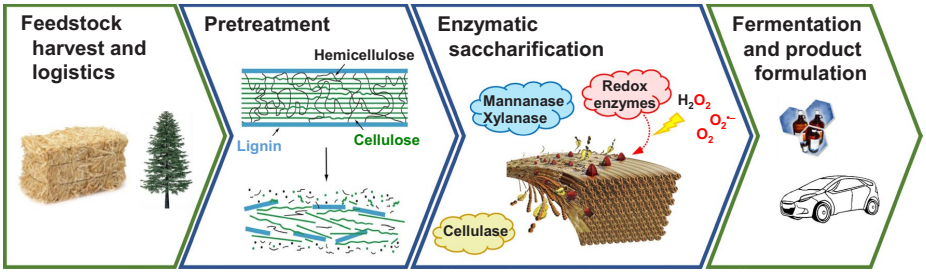

Fig. 1 The main steps of the conversion of lignocellulosic biomass to ethanol. Depending on the choice of microorganism in the fermentation step, a range of different fuels and chemicals may be produced

pretreated feedstock and on the interactions between these components in enzyme mixtures. Finally, we will address the limitations of today's cellulase cocktails and discuss possible strategies for their improvement.

Pretreatment technologies and their effect on the feedstock

A broad range of pretreatment technologies is available to enhance accessibility of lignocellulosic biomass to enzymes and hence promote saccharification, as reviewed by Yang and Wyman [389], Sun et al. [328] and Cantero et al. [50]. Among these, wet oxidation [307], hydrothermal pretreatment [270], steam explosion [44, 275], dilute acid treatment [252], ammonia fiber expansion (AFEX) [16], sulfite pulping [301, 377] and methods based on the use of ionic liquids and organic solvents [398] are the major technologies that have been used at demonstration or industrial scale over the past years. The choice of pretreatment depends on the type of feedstock as well as on the spectrum of desired end products [95, 301]. Hydrothermal pretreatment as well as AFEX and ammonium recycle percolation (ARP) technologies cause cellulose decrystallization, some hydrolysis of hemicellulose as well as lignin removal [18] and are primarily used for grass-type biomass (corn stover, switch grass), while steam explosion and alkaline and sulfite pulping can also be used for woody biomass (e.g., poplar and spruce). Recent improvements aim at reducing saccharification costs and include the following: (1) combined removal of lignin and hemicellulose prior to mechanical refining [54, 193, 388]; (2) restructuring native cellulose to the more accessible allomorph cellulose III in a low moisture extractive ammonia (AE) process [78]; and (3) the use of biomass-derived solvents for biomass pretreatment [179, 223, 322]. As an example, a pretreatment process recently developed at NREL [193], which uses a counter-current alkaline deacetylation [194] followed by mechanical defibrillation of the feedstock, allows enzymatic saccharification at high consistency, and the resulting hydrolysate is highly fermentable.

While some pretreatment technologies aim to increase plant cell wall accessibility via reorganization of plant cell wall polymers without removal of matrix polymers (AFEX, ARP), other technologies increase enzymatic accessibility of cellulose via fractionation of the biomass by separating lignin (e.g., alkali and sulfite pulping), hemicellulose (steam explosion) or both (ionic liquid or organosolv pretreatment) from cellulose. Detailed analysis of pretreated biomass with glycome profiling and immunolabeling of plant cell wall polymers indicate that not even the most efficient pretreatment technologies, such as hydrothermal pretreatment [86, 397], AFEX [264] and extractive ammonia pretreatment [13], can completely separate cellulose from the other cell wall polymers. Indeed, studies on the optimization of enzymatic biomass saccharification have revealed the need for a wide-spectrum enzyme cocktail, including cellulases and hemicellulases, to achieve complete saccharification of pretreated biomass, and the composition of the optimal enzyme cocktail depends on pretreatment and biomass type [21, 61, 168].

The active components of cellulase cocktails

Cellulolytic enzymes

In 1950, Reese et al. postulated that cellulose is degraded in a two-step process, the first step being the conversion of native, crystalline cellulose to shorter, accessible cellulose chains by a component called C_1 and the second step being the conversion of the now more accessible cellulose to oligomers and monomers by a component called C_x [291]. Over the years, the quest towards the isolation of the C_1 and C_x components from fungal secretomes (e.g., [130, 385]) led to the identification of the core set of fungal cellulose-active glycoside hydrolases (GHs), including

cellobiohydrolases (CBHs; cleaving off cellobiose from the cellulose chain ends), endoglucanases (EGs; cleaving cellulose chains in non-crystalline regions) and β-glucosidases (BGs; depolymerizing soluble cello-oligosaccharides liberated by CBHs and EGs) [386] (Fig. 2; Table 1). These GHs have been classified, based on sequence similarities, in the Carbohydrate Active enZymes (CAZy) database [219]. As an example, the model organism *T. reesei*, named after one of the pioneers of cellulase research, Elwyn T. Reese, secretes two CBHs, *Tr*Cel7A (formerly CBH I; a reducing end-specific CBH belonging to family GH7) and *Tr*Cel6A (formerly CBH II; a non-reducing end-specific CBH belonging to family GH6), four EGs, named *Tr*Cel7B (formerly EG I), *Tr*Cel5A (formerly EG II or, in the very early days, also EG III), *Tr*Cel12A (formerly EG III), *Tr*Cel45A (formerly EG V) and four BGs, *Tr*Cel3A (formerly Bgl1), *Tr*Cel3B, *Tr*Cel3F and *Tr*Cel3G [1, 231]. Two additional enzymes in the *T. reesei* secretome were initially annotated as EGs, namely *Tr*Cel61A (originally EG IV) [172] and *Tr*Cel61B (originally EG VII), but it is now clear that these enzymes are not EGs but lytic polysaccharide monooxygenases (LPMOs), as discussed below.

Although there have been some early indications that oxidative processes contribute to cellulose conversion [99], cellulose decomposition was thought, for a long time, to occur primarily through the action of hydrolytic enzymes. The breakthrough came in 2010 with the discovery of oxidative polysaccharide degradation by enzymes that were previously classified as CBM33s (chitin-binding proteins in bacteria) and GH61s (EGs in fungi) [351]. Today these enzymes are called lytic polysaccharide monooxygenases (LPMOs) and have been reclassified as Auxiliary Activity (AA) families 10 and 9, respectively, in the CAZy database [212]. Over the past decade, several LPMO families

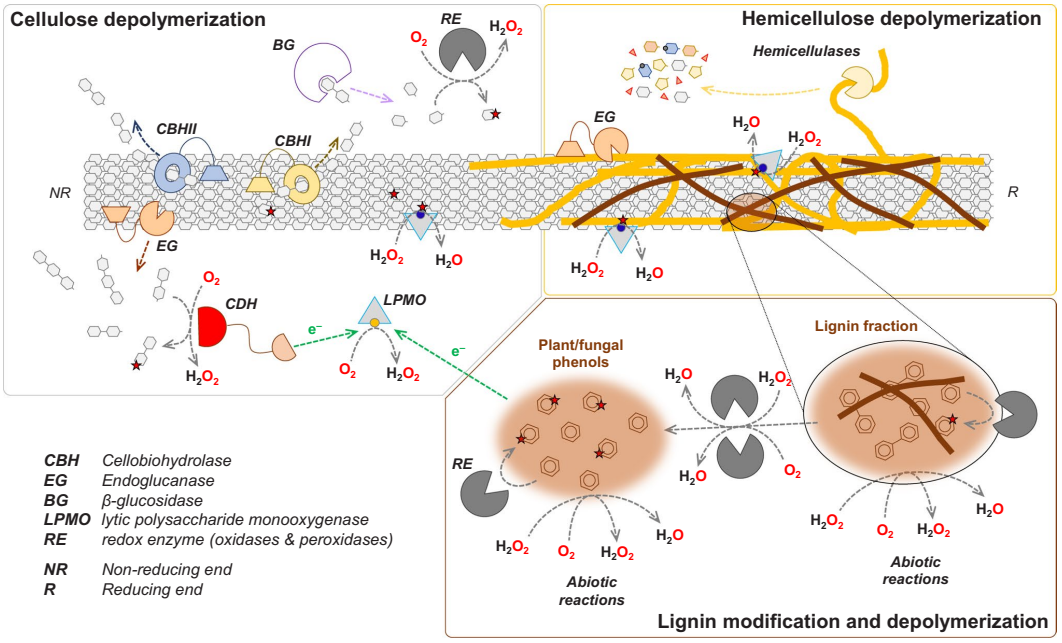

Fig. 2 Schematic view of a cellulose fibril covered with hemicellulose (orange) and lignin (brown) and key enzymes involved in the depolymerization of plant cell wall polysaccharides. The non-reducing (NR) and reducing (R) ends of the cellulose chains are marked. Stars indicate oxidation catalyzed by LPMOs (triangles) or other redox enzymes (RE, grey). Orange spheres depict Cu(II) and blue spheres depict Cu(I) in the active site of LPMOs. Interactions between hydrolytic and redox enzymes are indicated. For simplicity, the multitude of hemicellulose-active enzymes, including, e.g., debranching enzymes, are indicated as "hemicellulases", while lignin-active enzymes are referred to as redox enzymes ("RE"). Note that fungal secretomes may contain a variety of redox enzymes acting on oligosaccharides and monosugars that are released from cellulose or hemicellulose, as indicated in the "Cellulose depolymerization" panel. Also note that some LPMOs and EGs can act on the hemicellulose fraction, as indicated in the "Hemicellulose depolymerization" panel. A more comprehensive variant of this figure can be found in [39], and a more complete list of enzyme types is provided in Tables 1 and 2: *BG* β-glucosidase, *CBHI* cellobiohydrolase I, *CBHII* cellobiohydrolase II, *CDH* cellobiose dehydrogenase, *EG* endoglucanase, *LPMO* lytic polysaccharide monooxygenase, *RE* redox enzyme (oxidases and peroxidases)

Table 1 Plant cell wall polysaccharide-active enzymes of fungal origin that may be present in cellulase cocktails

Enzyme name	CAZy	EC	Mode of action	Example[i]
Cellulases				
Cellobiohydrolase (CBH)	GH7	3.2.1.176	Cleaving off cellobiose from the reducing end of cellulose chains	*Tr*Cel7A from *T. reesei* [231]
	GH6	3.2.1.91	Cleaving off cellobiose from the non-reducing end of cellulose chains	*Tr*Cel6A from *T. reesei* [231]
Endo-β-1,4-glucanase (EG)	GH5	3.2.1.4	Cleaving β-(1 → 4)-linkages in cellulose chains in non-crystalline regions (activity on hemicelluloses has been observed for some)	*Tr*Cel5A from *T. reesei* [231]
	GH7			*Tr*Cel7B from *T. reesei* [231]
	GH12			*Tr*Cel12A from *T. reesei* [231]
	GH45			*Tr*Cel45A from *T. reesei* [231]
β-glucosidase (BG)	GH3	3.2.1.21	Cleaving off D-glucose from the non-reducing end of oligosaccharides	*Tr*Cel3A (Bgl1) from *T. reesei* [231]
Hemicellulases				
Xyloglucanase	GH12 (EG)[a]	3.2.1.151	Cleaving β-(1 → 4)-linkages in xyloglucan chains	*Tr*Cel12A from *T. reesei* [393]
	GH74			*Tr*Cel74A from *T. reesei* [231]
Endo-β-1,4-xylanase	GH10	3.2.1.8	Cleaving β-(1 → 4)-linkages in xylan chains	*Tr*Xyn10A from *T. reesei* [231]
	GH11			*Tr*Xyn11A from *T. reesei* [231]
	GH7 (EG)[a]			*Tr*Cel7B from *T. reesei* [15]
Endo-β-1,4-mannanase	GH5	3.2.1.78	Cleaving β-(1 → 4)-linkages in glucomannan main chain	*Tr*Man5A from *T. reesei* [339]
	GH26			*Pa*Man26A *P. anserina* [69]
	GH134			*An*Man134A from *A. nidulans* [319]
	GH5 (EG)[a]			*Tr*Cel5A from *T. reesei* [173]
	GH7 (EG)[a]			*Tr*Cel7B from *T. reesei* [173]
	GH45 (EG)[a]			*Tr*Cel45A from *T. reesei* [173]
β-xylosidase	GH3	3.2.1.37	Cleaving off unsubstituted D-xylose from the non-reducing end of xylo-oligosaccharides	*Tr*Xyl3A (Bxl1) from *T. reesei* [231]
β-mannosidase	GH2	3.2.1.25	Cleaving off unsubstituted D-mannose from the non-reducing end of glucomanno-oligosaccharides	*An*Mnd2A from *A. niger* [3]
Hemicellulose debranching enzymes				
α-arabinofuranosidase	GH43	3.2.1.55	Cleaving off L-arabinosyl substitutions from xylans and xylo-oligosaccharides	HiAraF (GH43) from *H. insolens* [332]
	GH51			*An*AbfA (GH51) from *A. niger* [276]
	GH54			*Tr*Abf1 (GH54) from *T. reesei* [229]
	GH62			*Tr*Abf2 (GH62) from *T. reesei* [20]
α-galactosidase	GH27	3.2.1.22	Cleaving off α-(1 → 6)-linked D-galactosyl substitutions from glucomannan and glucomanno-oligosaccharides	*Tr*Agl1 (GH27) from *T. reesei* [228]
	GH36			*Tr*Agl2 (GH36) from *T. reesei* [228]

Table 1 (continued)

Enzyme name	CAZy	EC	Mode of action	Example[i]
α-glucuronidase	GH67 GH115	3.2.1.139, 3.2.1.131	Cleaving off α-(1→2)-linked D-glucuronic acid (3.2.1.139) or 4-*O*-methyl-D-glucuronic acid (3.2.1.131) sidechains of xylans and xylo-oligosaccharides	*At*AguA (GH67) from *A. tubingensis* [85] *Sc*Agu1 (GH115) from *S. commune* [56]
Deacetylases (incl. acetyl xylan esterase and acetyl mannan esterase)	CE1-6 and CE16[b]	3.1.1.6, 3.1.1.72, 3.1.1.–[e]	Hydrolysis of acetyl groups from various positions in xylans and xylo-oligosaccharides (3.1.1.6 and 72) and/or in glucomannans and glucomanno-oligosaccharides (3.1.1.-)	*Tr*Axe1 (CE5) [316] and *Tr*Axe2 (CE16) [214] from *T. reesei*; *Aw*AXE (CE1) from *A. awamori* [187]; *Np*BnaII (CE2) and *Nc*BnaIII (CE3), and *Nc*BnaI (CE6) from *N. patriciarum* [79] *Vv*AXEII (CE4) from *V. volvacea* [218]; *Ao*AGME from *A. oryzae* [341][j]
Feruloyl esterase	CE1	3.1.1.73, 3.1.1.–[f]	Cleaving off hydroxycinnamoyl groups esterifying arabinosyl substitutions of xylan backbone or lignin	*An*FaeA from *A. niger* [103] *Nc*FaeD from *N. crassa* [354]
Glucuronoyl esterase (GE)	CE15	3.1.1.–[g]	Cleavage of ester bonds between lignin alcohols and (4-*O*-methyl-D-glucuronic acid substitutions of xylan backbone	*Cu*GE from *C. unicolor* [246]
Lytic polysaccharide monooxygenase (LPMO)	AA9	1.14.99.54	Cleavage of cellulose chains with oxidation at the C1 carbon	*Tt*AA9E from *T. terrestris* [134]
		1.14.99.56	Cleavage of cellulose chains with oxidation at the C4 carbon	*Nc*AA9C from *N. crassa* [7]
		1.14.99.54, 1.14.99.56	Cleavage of cellulose chains with oxidation at the C1 or C4 carbon	*Ta*AA9A from *T. aurantiacus* [284]
		1.14.99.–[h]	Oxidative cleavage of β-(1→4)-linkages in xyloglucan chains (C1- and/or C4-oxidation)	*Nc*AA9C from *N. crassa* [7] *Ta*AA9A from *T. aurantiacus* [272]
		1.14.99.–[e]	Oxidative cleavage of xylan	*Mt*AA9A (MYCTH_85556) from *M. thermophila* [116]
	AA10[c]	1.14.99.54	Cleavage of cellulose chains with oxidation at the C1 carbon	*Sc*AA10C from *S. coelicolor* [112][c]
		1.14.99.53	Oxidative cleavage of chitin (C1-oxidation)	*Sm*AA10A from *S. marcescens* [351][c]
		1.14.99.54, 1.14.99.56, 1.14.99.53	Cleavage of cellulose chains with oxidation at the C1 or C4 carbon and oxidative cleavage of chitin (C1-oxidation)	*Sm*AA10B from *S. coelicolor* [109][c]
	AA11	1.14.99.53	Oxidative cleavage of chitin (C1-oxidation)	*Ao*AA11 from *A. oryzae* [139]

Table 1 (continued)

Enzyme name	CAZy	EC	Mode of action	Example[i]
	AA13	1.14.99.55	Oxidative cleavage of starch	*Nc*AA13 from *N. crassa* [371]
	AA14	1.14.99.–[e]	Oxidative cleavage of xylan	*Pc*AA14B from *P. coccinea* [68]
	AA15[d]	1.14.99.54	Cleavage of cellulose chains with oxidation at the carbon C1	*Td*AA15A from *T. domestica* [304][d]
	AA16	1.14.99.54	Cleavage of cellulose chains with oxidation of carbon C1	*Aa*AA16 from *A. aculeatus* [105]

The main CAZy families, the EC number and the mode of action regarding plant cell wall degradation are listed for each activity. Oxidoreductases other than LPMOs are listed in Table 2

[a]This enzyme is primarily known as endoglucanase but has a notable and potentially important side activity on hemicellulose

[b]Deacetylases are discussed together because there is variation in reported substrate preference and specificity among deacetylases belonging to the same CE families, and because the substrate preference (e.g., xylan, glucomannan, pectin or chitin) and/or specificity (deacetylation of e.g., xylosyl, glucosyl or mannosyl residues at position 2, 3 or 6) remains to be identified for most deacetylases. Of note, including deacetylases with complementary activities in cellulase cocktails is of high importance

[c]AA10 LPMOs are rarely found in fungi and are included for the sake of completion; none of the putative fungal AA10 LPMOs have been characterized, and the examples all refer to bacterial enzymes

[d]AA15 LPMOs have not been identified in fungi and are included for the sake of completion; the example refers to an arthropod enzyme

[e]EC number not created yet; no provisional EC number

[f]EC number not created yet; provisional EC number: 3.1.1.B10

[g]EC number not created yet; provisional EC number: 3.1.1.B11

[h]EC number not created yet; provisional EC number: 1.14.99.B11

[i]Strain abbreviations: *A. aculeatus*, *Aspergillus aculeatus*; *A. awamori*, *Aspergillus awamori*; *A. nidulans*, *Aspergillus nidulans*; *A. niger*, *Aspergillus niger*; *A. oryzae*, *Aspergillus oryzae*; *A. tubingensis*, *Aspergillus tubingensis*; *C. unicolor*, *Cerrena unicolor*; *H. insolens*, *Humicola insolens*; *M. thermophila*, *Myceliophthora thermophila*; *N. patriciarum*, *Neocallimastix patriciarum*; *N. crassa*, *Neurospora crassa*; *P. anserina*, *Podospora anserina*; *P. coccinea*, *Pycnoporus coccinea*; *S. coelicolor*, *Streptomyces coelicolor*; *S. marcescens*, *Serratia marcescens*; *S. commune*, *Schizophyllum commune*; *T. aurantiacus*, *Thermoascus aurantiacus*; *T. domestica*, *Thermobia domestica*; *T. reesei*, *Trichoderma reesei*; *T. terrestris*, *Thielavia terrestris*; *V. volvacea*, *Volvariella volvacea*

[j]The CAZy family for this enzyme has yet to be identified

have been described, and, as of today, families AA9-11, AA13-14 and AA16 comprise fungal LPMOs. AA15 type LPMOs have not been identified in fungi. Fungal LPMOs of the AA10 type are very rare and, while bacterial AA10s have been intensely studied, none of the putative fungal AA10s have been characterized. LPMOs contain a single copper co-factor, the reduction of which is crucial for the LPMO reaction [284, 351]. These enzymes catalyze the oxidative cleavage of β-1,4-glycosidic bonds of recalcitrant polysaccharides, either in a monooxygenase reaction using molecular O_2 and a reductant [351] or in a peroxygenase reaction using H_2O_2 [37, 38] (Fig. 3).

Importantly, the monooxygenase paradigm entails that reducing equivalents are being consumed by the LPMO in each catalytic cycle, whereas the peroxygenase reaction only requires priming amounts of reductant to reduce the LPMO to its catalytically active Cu(I) state (Fig. 3). It has been shown that the reducing power needed by LPMOs can be delivered in many ways, including a wide variety of small molecule reductants, such as ascorbic acid [351], phenolic compounds, including compounds derived from lignin and plant biomass in general [114, 190, 381], as well as certain redox enzymes [121, 190, 206, 274] (as reviewed by [39, 117]). Both the catalytic mechanism of LPMOs and the relative importance of the O_2-driven and H_2O_2-driven reactions

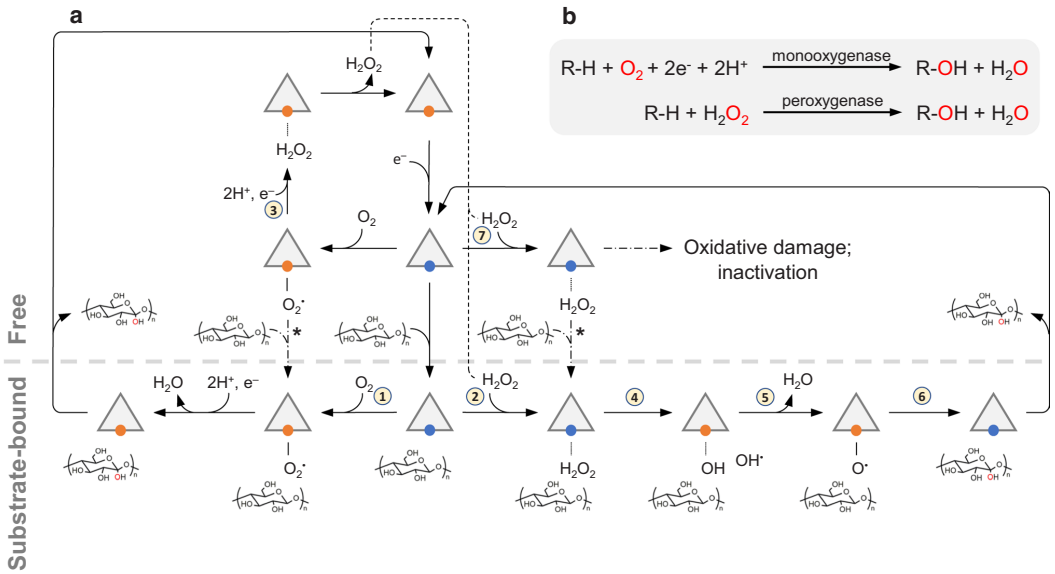

Fig. 3 Possible reaction schemes for LPMO-catalyzed cleavage of glycosidic bonds. The triangles represent the LPMO, and the small spheres the active-site copper. Orange spheres depict Cu(II) and blue spheres depict Cu(I). The bottom left of panel **a** shows the O_2-dependent monooxygenase reaction (1) and the bottom right of panel **a** shows the H_2O_2-dependent peroxygenase reaction (2). The upper part of panel **a** shows reactions that may occur in the absence of a polysaccharide substrate. The order of binding events is not fully resolved and the figure shows two scenarios, where the less likely one is labeled by an asterisk. Current data support formation of a ternary complex and do not support a ping-pong mechanism [163, 200]. It is interesting to note that reduction of the LPMO promotes substrate binding [188, 201] and could thus promote ternary complex formation. A scenario where the LPMO remains closely associated with the substrate in between consecutive catalytic cycles is conceivable. Panel **b** shows the simplified reaction schemes for the proposed LPMO reactions. Note that several reaction mechanisms have been proposed for both the monoxygenase reaction [28, 235, 374] and the peroxygenase reaction [37] and that the figure shows one of several possible scenarios for each reaction. The figure also shows the uncoupling reaction with O_2 that leads to formation of H_2O_2 (3; top left). In the H_2O_2-dependent reaction mechanism, step 4 indicates homolytic cleavage of the O–O bond of H_2O_2, for which experimental and computational evidence is available [38, 163, 375]. One possible outcome is the subsequent formation of an oxyl intermediate (step 5), which has often been proposed as the hydrogen-abstracting intermediate in studies on LPMO catalysis. In this case, hydrogen abstraction would be followed by binding of the resulting hydroxyl to the substrate radical, in an oxygen-rebound mechanism (step 6). Hydroxylation leads to destabilization of the glycosidic bond and will be followed by spontaneous bond cleavage ([274]; not shown). While homolytic cleavage of H_2O_2 is supported by recent experimental evidence [163], alternative scenarios are thinkable [37, 163, 375]. Step 7 shows the reaction of a reduced LPMO with H_2O_2 in the absence of substrate (top right), which can damage the enzyme and lead to inactivation. It is worth noting that there is at least one additional example of an enzyme, in this case a non-heme mono-iron epoxidase, that was originally thought to be an oxidase (i.e., using O_2) and that later turned out to use H_2O_2 [376]

are the subject of debate and current research, as recently reviewed in [39, 60].

Since the postulation of the C_1–C_x theory for cellulose depolymerization by Reese et al. [291], the nature of the C_1 factor has been interpreted in a number of ways. First, cellobiohydrolases were thought to act as C_1 factor [129]. It has been suggested that CBHs break non-covalent linkages between adjacent cellulose chains in crystalline cellulose since they thread a single cellulose chain into their active site cleft (or even tunnel) and, thus, are potentially capable of extracting a longer piece of cellulose chain out of its crystalline context [122, 182]. While lifting a single cellulose chain (likely 6 or more glucose units) away from the crystalline lattice, i.e., decrystallization of cellulose, carries an energy penalty, strong binding interactions between the enzyme and the cellulose, which relate to the processive nature of CBHs, could make such decrystallization energetically possible (see also below). Later, Arantes and Saddler proposed that carbohydrate-binding modules (CBMs), such as the one attached to the most studied CBH, *Tr*Cel7A, and expansin-like proteins, such as the Swo1 swollenin protein that induces swelling of cellulose [305], may fulfil the role of the C_1 factor [10]. The discovery of LPMOs has led to the speculation that these enzymes may in fact be the long-sought-after C_1 factor [142, 245, 351]. This hypothesis is supported by multiple studies showing that LPMOs belonging to various AA families induce fibrillation of cellulose fibers [149, 352, 364].

Of the *T. reesei* cellulases, the CBH *Tr*Cel7A has gained the most attention, primarily because it is the most abundant enzyme in the secretome, comprising close to 60% of the cellulolytic proteins [126]. The crystal structure of the catalytic domain of *Tr*Cel7A reveals a tunnel-shaped active site [89], which can accommodate ten glucosyl units [64, 88]. The long substrate-binding tunnel of *Tr*Cel7A enables strong interactions with a single cellulose chain and contributes to the processive mode of action of this enzyme [26, 181, 182], as visualized by Igarashi et al. using high-speed atomic force microscopy [151]. Processivity is a key attribute of CBHs that makes them especially powerful in depolymerizing the highly compact structure of crystalline cellulose [26, 338, 362]. On the other hand, processivity leads to stalling of CBHs when their path is blocked by other enzymes or substrate-derived obstacles [73, 113, 152, 155, 199]. Furthermore, it has been claimed that the strong binding energies associated with processivity, in particular reflected in low off-rates [74, 198], make processive GHs intrinsically slow, as has been nicely demonstrated for processive chitinases [141, 394, 395].

Contrary to the CBHs, with their deep substrate-binding clefts, or even tunnels, cellulose-active LPMOs have a flat substrate-binding and catalytic surface, which is optimized for attacking surfaces such as those found in cellulose crystals [171, 350, 351]. Unlike CBHs and other GHs, LPMOs cannot use binding energy to distort the substrate towards the transition state for hydrolytic glycoside bond cleavage. Thus, LPMOs employ powerful oxidative chemistry, allowing them to cleave the β-1,4-glycosidic bonds of cellulose without the need to remove a cellulose chain from the crystalline lattice. Some LPMOs are known to act on non-crystalline substrates [7, 102, 154], and the most commonly used substrate for assaying the activity of cellulose-active LPMOs is phosphoric-acid swollen (so, non-crystalline) cellulose. Still, the ability of LPMOs to attack crystalline and other recalcitrant and insoluble polysaccharide structures [68] is well documented [96, 351, 364] and likely comprises the most important role of these enzymes in biomass conversion.

Hemicellulolytic enzymes

Depending on the type of biomass and pretreatment technology, pretreated biomass contains, in addition to cellulose, varying amounts of linear and branched polysaccharides, including the hemicelluloses xylan, glucomannan and xyloglucan, as well as pectin, all of which adhere to cellulose fibers, forming a complex three-dimensional matrix [323]. These polysaccharides can form multiple substructures, and while many hemicelluloses are relatively easy to degrade, a fraction of these polysaccharides will form recalcitrant co-polymeric substructures that may hamper cellulose degradation [47, 261, 392]. Due to the high complexity of these plant polysaccharides, a variety of enzyme activities are needed for their complete breakdown (Table 1). The most studied hemicellulose-active enzymes are xylan- and glucomannan-specific enzymes. These hemicellulases include GHs that cleave the polysaccharide main chain, i.e. endo-β-1,4-xylanases (shortly xylanases) and endo-β-1,4-mannanases (shortly mannanases), as well as debranching enzymes that remove substitutions from the polysaccharide backbone (e.g., deacetylases, arabinosidases and galactosidases). These enzymes and their potential uses have been reviewed by Malgas et al. [224, 227]. Interestingly, recent studies indicate that LPMOs belonging to class AA14 may be tailored to specifically act on recalcitrant xylan coating cellulose fibers [68] (Fig. 2).

In addition to hemicellulases, some EGs and AA9 LPMOs may also contribute to hemicellulose conversion because they are capable of cleaving the polysaccharide backbones of some, or even a wide range, of hemicellulosic polysaccharides, including xyloglucan, xylan and/or glucomannan [7, 102, 116, 150, 183, 320, 366] (Fig. 2, Table 1). While promiscuous endoglucanases [366] and some of the hemicellulolytic LPMOs cleaving mixed-linkage glucans, xyloglucan and glucomannan [7, 102, 183, 251, 272, 320], are active on isolated hemicelluloses, xylan-active AA9 (and

also AA14) LPMOs [68, 114, 116, 150] require xylan being complexed with cellulose. A likely reason for this is that insoluble forms of hemicelluloses associated with cellulose adopt different conformations than their soluble forms [47]. Consequently, screening for enzyme activity on natural substrates or pretreated biomass instead of model substrates, such as microcrystalline or amorphous cellulose and isolated hemicelluloses, may be a prerequisite for accurately describing substrate specificities, or for detecting enzyme activity in the first place [68].

An evolutionary advantage for substrate promiscuity for EGs and LPMOs could be the ability to cleave recalcitrant fractions of xyloglucan, xylan and glucomannan that adhere to cellulose fibers. As an example, *Tr*Cel7B is active on xylan [15], glucomannan [239] and xyloglucan [366]. In terms of promiscuity among EGs and LPMOs, the fact that GH7 EGs (such as *Tr*Cel7B), and potentially also some AA9 LPMOs, can act on both xylan and glucomannan likely contributes to their importance in enzyme cocktails for biomass breakdown [61, 168, 300, 355]. It is noteworthy that the activity of *Tr*Cel7B from *T. reesei* on xylan is comparable to, if not higher than, its activity on cellulose [15]. Xylans are abundant in all types of lignocellulosic plant biomass (i.e., grasses, hardwood and softwood), emphasizing the importance of xylan-active EGs and CAZymes in general in enzyme cocktails, irrespective of the origin of the feedstock. Most importantly, inclusion of CAZymes with broad substrate specificities will help in designing universal enzyme cocktails for the breakdown of a broad range of biomass.

Complementarily to the action of enzymes converting hemicellulose polymers to shorter fragments, debranching enzymes are needed to enable the complete saccharification of hemicellulosic oligomers by β-xylosidases and β-mannosidases [224, 227]. Some debranching enzyme activities may be of particular importance as they cleave covalent linkages to lignin [157]. Substitutions of xylans include hydroxycinnamoyl and glucuronoyl groups, which have been shown to take part in the formation of covalent linkages between lignin and xylan. Enzymes potentially acting on lignin–hemicellulose bonds include feruloyl esterases, cleaving off hydroxycinnamoyl (including feruloyl, *p*-coumaroyl, and cinnamoyl) groups from arabinosyl substitutions of the xylan backbone [71], and glucuronoyl esterases, cleaving off lignin alcohols having ester bonds with (methyl)-glucuronic acid substitutions of the xylan backbone [101, 243, 246]. These enzymes have received considerable attention as enzymatic cleavage of lignin–polysaccharide bonds potentially has a dual positive effect in biomass conversion: (1) improvement of enzymatic accessibility of plant cell wall polysaccharides and (2) removal of hemicellulose moieties from the residual lignin. The relevance of these enzymes for complete biomass saccharification is emphasized in a recent study by Mosbech et al., showing that a glucuronoyl esterase from *Cerrena unicolor*, in combination with a GH10 xylanase, is able to completely remove xylan moieties from birchwood lignin [246].

Debranching enzymes and deacetylases are especially important in biomass decomposition because hemicelluloses coating cellulose microfibrils, in particular xylan and glucomannan, are known to be acetylated and substituted with glucuronic acid or galactose [46, 125, 392]. Removal of these substitutions changes cellulose–hemicellulose interactions and may decrease the recalcitrance of the feedstock [265]. On the other hand, removal of substitutions from xylan and glucomannan polymers that are not directly associated with cellulose microfibrils may decrease their solubility in water and lead to the adsorption of linear, unsubstituted hemicellulose fragments onto cellulose fibers [165, 195, 379]. While these hemicelluloses can be removed by xylanases and mannanases, they will limit cellulose accessibility [379, 380]. In addition to acting on hemicelluloses, acetyl esterases may also act on lignin and change its properties [265], but the implications of this effect, and of the effects of deacetylating enzymes in general remain to be studied.

Other oxidoreductases in biomass conversion

In addition to GHs and LPMOs, fungal secretomes are rich in oxidoreductases, including cellobiose dehydrogenases (CDHs; belonging to family AA3_1 in CAZy), lignin-active laccases (family AA1) and peroxidases (family AA2), copper-radical oxidoreductases (family AA5) and multi-copper oxidoreductases (family AA3). A detailed overview of these enzymes and potential interactions between them is provided in a recent review by Bissaro et al. [39]. Some of these oxidoreductases have been shown to directly (CDH) or indirectly (laccase and polyphenol oxidase) interact with LPMOs (Fig. 2; Table 2). CDHs can reduce the active-site copper of LPMOs directly via their AA8 cytochrome domain [335], thus fueling the LPMO reaction, and may also contribute by generation of the LPMO co-substrate H_2O_2 [189]. Two polyphenol oxidases have been shown to promote LPMO reactions because they hydroxylate methylated or non-methylated monophenols (including lignin monomers), which thus become better reductants for LPMOs [115]. Alternatively, laccase treatment of lignin, which as such is known to be able to drive LPMO reactions (see above), has led to increased LPMO activity [42, 269]. Perna et al. showed that the observed effect is due to increased H_2O_2-production by reactions involving laccase-modified lignin [269]. For the successful exploitation of these effects in biomass conversion, however, further research is needed, addressing, for example, the interaction of lignin-active oxidoreductases with lignin, as well as the actual flow of electrons, the

Table 2 Fungal oxidoreductases that may be present in commercial cellulase mixtures and that may affect LPMO activity

Enzyme name	CAZy family/EC number	Proposed mode of interaction	Examples[h]
Cellobiose dehydrogenase (CDH)	AA3_1 1.1.99.18	Reduction[a] and in situ generation of H_2O_2[b,c]	*Hi*CDH from *H. insolens* + *Ta*AA9A from *T. aurantiacus* [206] *Mt*CDH-1 from *M. thermophila* + *Tt*AA9E from *T. terrestris*, *Mt*AA9E (MYCTH_85556) from *M. thermophila*, and *Tr*AA9A from *T. reesei* [45] *Mt*CDH-2 from *M. thermophila* + *Nc*AA9M [274], 9D, and 9E [27, 274] and *Nc*AA13 [370] from *N. crassa* and *Mt*AA9E (MYCTH_79765) [131] and variants of *Mt*AA9D (MYCTH_92668) from *M. thermophila* [324] *Mt*CDH from *M. thermophilum* + *Nc*AA9F [335], 9C [43, 154, 273], 9A, and 9D [273] from *N. crassa* and *Ta*AA9A from *T. aurantiacus* [272] *Mt*CDH from *M. thermophilum* + *Sc*AA10C from *S. coelicolor* and *Sm*AA10A [37, 220] and variants thereof [221] from *S. marcescens* Variants of *Mt*CDH from *M. thermophilum* + *Nc*AA9C from *N. crassa* and *Sm*AA10A from *S. marcescens* [189][c] *Nc*CDH IIA + *Nc*AA9C [67, 104, 180, 190, 330], 9F [180, 190, 335], 9E, and 9J [180, 190] from *N. crassa* and *Ps*AA9A and 9B from *Pestalotiopsis* sp. [263] *Nc*CDH IIB + *Nc*AA9C [180, 190, 330], 9E, 9F, and 9J [180, 190] from *N. crassa* and *Ps*AA9A and 9B from *Pestalotiopsis* sp [263] *Pa*CDHB + *Pa*AA9A, 9D, 9E, 9F, 9G, and 9H from *P. anserina* [31] *Pc*CDH from *P. cinnabarinus* + *Pa*AA9A and 9B from *P. anserina* [34] *Tt*CDH + *Tt*AA9E from *T. terrestris* [206]
		Reduction of redox mediators that can affect LPMO reactions[d]	The AA3_1 domain of *Mt*CDH from *M. thermophilum* + *Nc*AA9C from *N. crassa* [190]
Pyranose dehydrogenase (PDH), PQQ-dependent	AA12 1.–.–.–	Reduction[a] and, possibly[e], in situ generation of H_2O_2[f]	*Cc*PDH from *C. cinerea* + *Nc*AA9C and 9F [357] and *Nc*AA9A and 9D [273] from *N. crassa*
Pyranose dehydrogenase (PDH), FAD-dependent	AA3_2 1.1.99.29	Reduction of redox mediators that can affect LPMO reactions[d]	*Am*PDH from *A. meleagris* + *Nc*AA9C from *N. crassa* [190][i]
Glucose dehydrogenase GDH	AA3_2 1.1.5.9	Reduction of redox mediators that can affect LPMO reactions[d] and, possibly[e], in situ generation of H_2O_2	GDH from *G. cingulata* + *Nc*AA9C from *N. crassa* [190][i] GDH from *P. cinnabarinus* + *Pa*AA9E from *P. anserina* [121]
Glucose 1-oxidase (GOx)	AA3_2 1.1.3.4	Reduction of redox mediators that can affect LPMO reactions[d]	*An*GOx from *A. niger* + *Nc*AA9C from *N. crassa* [190][i]
		In situ generation of H_2O_2[g]	*An*GOx from *A. niger* + *Sc*AA10C from *S. coelicolor* [37] *An*GOx from *A. niger* + *Nc*AA9C from *N. crassa* [104]

Table 2 (continued)

Enzyme name	CAZy family/EC number	Proposed mode of interaction	Examples[h]
Aryl-alcohol quinone oxidoreductase (AAQO)	AA3_2	Reduction[a] and, possibly[e], in situ generation of H_2O_2	AAQO1 and AAQO2 from *P. cinnabarinus* + *Pa*AA9E from *P. anserina* [121]
Aldose oxidase (AOx)	AA7 1.1.3.-	In situ generation of H_2O_2	*Mn*AOx from *M. nivale* + *Ta*AA9A from *T. aurantiacus* and Cellic CTec3 [266][j]
Laccase	AA1 1.10.3.2	Generation of H_2O_2 via lignin oxidation	Laccase from *T. versicolor*, *M. thermophila*, *G. lucidum*, and *Amycolatopsis* sp. + *Sm*AA10A from *S. marcescens* and *Nc*AA9C from *N. crassa* [269]
Polyphenol oxidase	(not in CAZy) 1.14.18.1	Activation of lignin for more efficient reduction[a] and/or in situ generation of H_2O_2[e]	*Ab*PPO from *A. bisporus* and *Mt*PPO7 from *M. thermophila* driving *Mt*AA9B (MYCTH_80312) from *M. thermophila* [115]
Versatile peroxidase	AA2 1.11.1.14	LPMO-generated H_2O_2 drives peroxidase activity	*Ps*VP from *Physisporinus* sp. + *Po*LPMO9A from *P. ostreatus* [213]
Catalase	(not in CAZy) 1.11.1.6	Preventing oxidative damage by keeping H_2O_2 concentrations low	Catalase from *T. aurantiacus* + *Ta*AA9A from *T. aurantiacus* and Cellic CTec3 [266, 312] Catalase from *C. glutamicum* + *Nc*AA9C from *N. crassa* [104]

The tested enzyme pairs and the (putative) modes of interaction between them are listed for each type of oxidoreductase

[a]The role and nature of the reduction step differs between catalytic scenarios, as outlined in the main text and Fig. 3 [37]. Reduction may be seen as a "priming event", i.e., activation of the LPMO for subsequent multiple H_2O_2-driven turnovers. Alternatively, in the O_2-driven scenario, two electrons need to be delivered per catalytic cycle

[b]Electron transfer from CDH to the active site copper of the LPMO is mediated by the AA8 cytochrome domain and has been observed in several studies, e.g., [190, 330, 335]. Alternatively, electrons may be transferred directly from the DH domain to O_2, leading to the generation of H_2O_2 [189]

[c]Reference [189] provides evidence showing that the ability of engineered CDH variants to drive LPMO reactions correlates with the ability of these variants to generate H_2O_2

[d]The role of redox mediators has been addressed in various studies and has so far only been linked to reduction of the LPMO. Redox mediators may also affect H_2O_2 levels in the reaction

[e]The production of H_2O_2 and its potential impact on the LPMO were not assessed, but it is conceivable that H_2O_2 production occurred under the conditions used

[f]The domain structure of *Cc*PDH is analogous to that of CDHs, suggesting that the two enzymes use similar mechanisms in driving LPMO reactions [357]

[g]GOx can generate H_2O_2, the co-substrate of LPMOs, but is unable to reduce LPMOs [37]

[h]Strain abbreviations: *A. bisporus*, *Agaricus bisporus*; *A. meleagris*, *Agaricus meleagris*; *A. niger*, *Aspergillus niger*; *C. cinerea*, *Coprinopsis cinerea*; *C. glutamicum*, *Corynebacterium glutamicum*; *G. cingulata*, *Glomerella cingulata*; *G. lucidum*, *Ganoderma lucidum*; *H. insolens*, *Humicola insolens*; *M. nivale*, *Microdochium nivale*; *M. thermophila*, *Myceliophthora thermophila*; *M. thermophilum*, *Myriococcum thermophilum*; *N. crassa*, *Neurospora crassa*; *P. anserina*, *Podospora anserina*; *P. cinnabarinus*, *Pycnoporus cinnabarinus*; *P. ostreatus*, *Pleurotus ostreatus*; *S. coelicolor*, *Streptomyces coelicolor*; *S. marcescens*, *Serratia marcescens*; *T. aurantiacus*, *Thermoascus aurantiacus*; *T. terrestris*, *Thielavia terrestris*; *T. versicolor*, *Trametes versicolor*

[i]The ability of the enzyme to reduce redox mediators that can affect LPMO reactions was tested; reactions with LPMO, i.e., the enzyme, redox mediator and LPMO, were not shown

[j]While this study showed in situ generation of H_2O_2, it did not show a beneficial effect of AOx on LPMO activity

generation and consumption of H_2O_2 and effects on both the LPMOs and other enzyme components.

Co-operativity between enzyme components

In order to gain a deeper understanding of the mechanisms behind enzymatic biomass decomposition, individual enzyme components have been studied alone (enzyme characterization studies) and in combination with other individual enzyme components (minimal enzyme cocktail studies), cellulase cocktails or fungal secretomes (supplementation or spiking studies). Already in the late 1970s, co-operativity (Fig. 4) between different cellulases became clear when Wood and McCrae showed that CBHs enhance swelling of cotton fibers by EGs [387]. Shortly thereafter, CBHs and EGs were described to exert a mutually positive effect on each other's action during cellulose hydrolysis [140]. In other words, it was demonstrated that these two enzymes act synergistically (Fig. 4). Since then, several types of synergism have been observed between cellulolytic enzymes: between CBHs and EGs [253], CBHs, EGs and cellulose-active AA9 LPMOs [134], and two cellulose-active AA10 LPMOs [109]. The mechanisms of synergies between cellulolytic enzymes have been in the focus of research on biomass degradation, especially for cellulose, using for example detailed kinetic models [155, 253, 373] and atomic force microscopy [96, 120, 152]. A classical interpretation of this synergy is that EGs generate new chain ends for CBHs, but recent studies have indicated that additional mechanisms need to be considered [41, 100, 155, 202, 257, 279]. In particular, it has been proposed that EGs may promote CBH activity by attacking amorphous regions in the cellulose

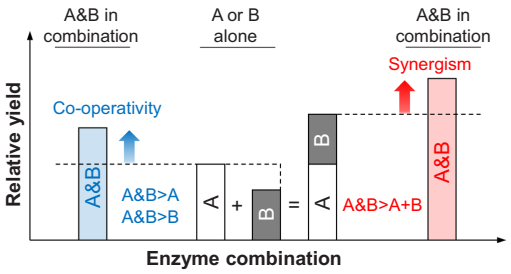

Fig. 4 Schematic representation of the difference between co-operativity and synergism between enzymes. Co-operativity between two or more enzymes implies that concomitant action of the enzymes gives saccharification yields that are higher than the yields obtained in reactions with individual enzymes (on the left, in blue). Synergism between enzymes implies that the concomitant action of the enzymes results in a yield that is higher than the sum of the yields obtained in reactions with the individual enzymes (on the right, in red)

that CBHs are unable to pass during processive action [155, 279].

Over the past decade, the interplay of LPMOs with hydrolases has gained considerable attention [11, 96, 97, 175, 248]. Studies with chitin-active [349] and cellulose-active [134, 238] LPMOs have shown that these enzymes promote the action of classical hydrolytic enzymes, and after the discovery of the catalytic activity of LPMOs [351], it became clear that the presence of reducing power promotes the LPMO effects. Indeed, Harris et al. observed that the boosting effect of an LPMO on cellulase action required the presence of other compounds in the biomass, most likely lignin-derived [134]. In retrospect, it is clear that these observations relate to the reducing power that is present in biomass but not in model cellulosic substrates such as Avicel [134, 143, 247]. In an important study, Eibinger et al. used confocal microscopy to show that a cellulolytic LPMO from *N. crassa* primarily acts on surface-exposed crystalline areas of the cellulose and that LPMO treatment promoted adsorption of a CBH, *Tr*Cel7A, to these crystalline regions, resulting in more efficient hydrolysis of these cellulose crystals [96]. Subsequent studies using real-time atomic force microscopy led to similar conclusions [97]. The work by Eibinger et al. provides evidence that at least some LPMOs cleave cellulose at crystalline areas and thus produce new chain ends, i.e. action sites, for CBHs. This highlights an important difference between LPMOs and EGs in terms of their mode of synergism with CBHs, since these enzymes cleave crystalline and amorphous parts of cellulose, respectively.

Notably, the oxidation at the terminal glucose molecules after LPMO action will have multi-faceted impact on CBHs that will depend partly on the directionality of CBHs and partly on the affinity of individual CBHs for the oxidized chain ends. One of the two new chain ends generated by an LPMO will be oxidized, and CBHs may vary in terms of how well they interact with such oxidized chain ends. Interestingly, molecular simulation studies on the oxidative cleavage of crystalline cellulose by LPMOs performed by Vermaas and colleagues indicated that C4-oxidized chain ends (i.e. oxidized at the non-reducing end) will be more readily hydrolyzed by non-reducing end-specific GH6 CBHs, such as *Tr*Cel6A [361].

Co-operativity between enzymes has also been studied in detail for degradation of various hemicelluloses [83], including xylan [224] and glucomannan [227], the most abundant hemicelluloses in lignocellulosic biomass. On hemicelluloses, synergism occurs primarily between enzymes hydrolyzing the polysaccharide main chain and debranching enzymes. For xylan depolymerization, examples include synergism between the following: a xylanase and an arabinosidase [186, 360], xylanases and a glucuronidase [85], xylanases and acetyl esterases [35], a GH11 xylanase and

a CE5 acetylxylan esterase [315, 316], a GH10 or GH11 xylanase and a CE1 feruloyl esterase [84, 103] and a GH10 xylanase and a CE15 glucuronoyl esterase [246]. In addition, synergism between a GH11 xylanase and an AA14 LPMO, both acting on the xylan backbone, has recently been observed [68]. Saccharification of glucomannan has been less studied because the plant cell walls of grasses and herbaceous plants, the more commonly used feedstocks for ethanol production, do not contain glucomannan. Examples of enzyme synergism in glucomannan degradation include the following: a mannanase and galactosidases [63, 228], a mannanase a galactosidase and two acetyl esterases [341], and a GH5 mannanase and a CE2 acetyl esterase [12].

Notably, studies on polysaccharide utilization loci in bacteria from the gut microbiota may provide further insight into the interplay of backbone-cleaving and debranching enzymes for compounds such as xyloglucan [208], pectin [222], xylan [297] and glucomannan [76, 204]. Since these polysaccharide utilization loci likely encode all enzymes needed for saccharification of a certain polysaccharide, they provide hints as to the preferred composition of enzyme cocktails for biomass saccharification containing fungal enzymes.

In natural biomass, cellulose, hemicelluloses (xyloglucan, xylan and/or glucomannan), pectin and lignin co-occur, and hence synergism of enzymes acting on different plant cell wall components can be anticipated to occur. Such "intermolecular synergism" has been described in the late 1990s for cellulases and xylanases acting on birch kraft pulp and for cellulases, xylanases and mannanases acting on spruce kraft pulp by Tenkanen et al. [340] and later for CBH and xylanase acting on pretreated corn stover by Selig et al. [316]. The interplay between cellulases and enzymes acting on hemicellulose has also been extensively studied by the Saddler group [144–146, 327]. Notably, cellulases, xylanases and mannanases work synergistically with each other on spruce chemical pulp not only in the initial phase of the saccharification Várnai [359] but also throughout the course of the reaction. Apparently, depolymerization of cellulose, xylan and glucomannan proceeds simultaneously throughout the process, indicative of a "peeling" type of synergism [355]. In a recent study, Nekiunaite and co-workers showed that cleavage of cellulose by a cellulose-active LPMO from *N. crassa* is inhibited by the presence of xyloglucan and that this inhibition is alleviated by adding a xyloglucan-active EG [251]. These findings point at the possible importance of promiscuous EGs [366] and LPMOs [7, 102, 114, 150] in the complete saccharification of lignocellulosic biomass. It seems clear that for the complete saccharification of any feedstock of interest, it is essential to identify key plant cell wall components that may hinder access to cellulose and other plant cell wall polysaccharides and to identify the corresponding carbohydrate-active enzymes (CAZymes) that cleave these.

Co-operativity or synergism?

It is important to note that the term synergism should be used with care. Synergism between two enzyme components occurs if the concomitant action of the two enzymes results in a higher yield than when summing up the yields obtained when using the individual components (Fig. 4). Synergism is best observed between pure enzymes using low enzyme dosages and short reaction times, i.e. staying in the initial linear phase of the saccharification reaction [9, 225, 355]. Using longer incubation times may mask positive effects of combining enzymes acting on the same plant cell wall polymer. This can happen when the concomitant action of the enzymes leads to faster saccharification, which can be observed in the initial phase, but does not lead to higher final conversion yields.

While carefully designed laboratory experiments addressing synergistic effects may give insights into the mechanism of interaction between a selection of individual enzyme components, understanding the importance of individual enzyme components in cellulase cocktails remains challenging. To elucidate the effect of individual enzyme components on the total conversion yield, studies on the development of minimal enzyme cocktails (i.e. optimizing blends of individual enzymes [21, 61, 168]) as well as spiking studies (i.e. partial replacement or supplementation of cellulase cocktails with an enzyme preparation [143, 146, 177, 250]) are used routinely. Such studies can lead to the identification of key enzyme components that are necessary for efficient saccharification of a feedstock. Since enzyme production costs (i.e., protein production costs) are an important factor in enzyme-based biorefining, it is important that the total protein loading is fixed in studies aimed at investigating enzyme co-operativity and identification of limiting activities [145]. A few examples of enzyme activities that may be limiting in the industrial conversion of lignocellulosic biomass are discussed below.

LPMOs and catalases

Using technical substrates (i.e. pretreated biomass) to test the performance of enzyme cocktails is essential for industrial relevance. This is exemplified by the early work of Harris et al., which indicated that LPMOs are active on lignocellulosic substrates (such as pretreated corn stover) but not on pure cellulose substrates [134]. An explanation for these initial findings only became clear after the discovery that LPMOs need electrons, which lignin can provide [114, 381]. Recent studies indicate that lignin has a dual function in LPMO activation: it is able to reduce the active site-copper

of LPMOs and to produce H_2O_2 in situ from O_2 [185, 269]. Importantly, lignin-active enzymes can affect the electron-donating and H_2O_2-generating abilities of lignin, providing possible links between polysaccharide- and lignin-degrading enzyme systems [42, 115, 269]. Another possible link between these systems is that LPMO-facilitated in situ production of H_2O_2 may be utilized by peroxidases to degrade lignin [213].

To employ LPMOs in the degradation of lignin-poor cellulosic substrates, it is necessary to supply the saccharification reaction with external reducing agents like ascorbic acid to activate the LPMOs [250]. For saccharification of cellulose-rich sulfite-pulped spruce, it has been shown that lignin-containing spent sulfite liquor can work as an electron donor [62, 65]. On the other hand, accumulating data confirm that the LPMO reaction can be driven by lignin remaining in the biomass after various pretreatments, including dilute-sulfuric acid pretreatment [134], steam explosion [250] or hydrothermal pretreatment [48, 185], although to varying extents [296]. Thus, while lignin may be inhibitory to cellulases due to unproductive enzyme binding [23, 32, 91, 260, 287, 288, 347] or shielding the polysaccharide [90, 191], it may be crucial for LPMO activity in certain experimental settings.

LPMO activity depends on supply of H_2O_2, either direct or indirect, i.e in situ production of H_2O_2 from O_2. The latter needs a much higher supply of reductant (Fig. 3) and may only be feasible when the feedstock is relatively rich in lignin. For substrates with low lignin content, direct supply of H_2O_2 works extremely well [248], also at demonstration scale [65]. For lignin-rich substrates, however, the benefits of direct addition of external H_2O_2 are less clear [248], presumably due to side-reactions occurring between added H_2O_2 and lignin [185]. In situ production of H_2O_2 may happen close to the enzyme, perhaps even on the enzyme, which will increase the likeliness that the generated H_2O_2 is indeed used by the LPMO rather than being consumed in side reactions between H_2O_2 and lignin.

A drawback of processes relying on in situ production of H_2O_2 is the lack of direct control over the amount of H_2O_2 produced, meaning that intermittently high concentrations of H_2O_2 (and other reactive oxygen species derived from H_2O_2) could be experienced, which may be damaging to the enzymes. Accumulation of H_2O_2 may be prevented by the use of catalases, which convert H_2O_2 to water. Indeed, a study by Scott et al. showed that inactivation of LPMO-containing cellulase blends was significantly reduced by addition of catalases [312]. Thus, a likely role of catalases, which are also present in fungal secretomes together with LPMOs [2], is to maintain low H_2O_2 levels in systems with in situ H_2O_2 generation (Table 2). Since catalases have K_m values for H_2O_2 in the millimolar range, while LPMOs have K_m values for H_2O_2 in the micromolar range [39, 200], LPMOs will still be active and not directly inhibited by the H_2O_2 consumption of the catalases. It should also be noted that abiotic factors will consume oxygen and generated reactive oxygen species during typical incubation conditions for enzymatic saccharification of lignocellulosic materials (as illustrated in Fig. 2), and many aspects of the reactions taking place are not yet fully understood [266].

Today's cellulase cocktails: what are the limitations and how to overcome these?

Commercial enzyme cocktails have been greatly improved since initial cocktails were launched on the market [160, 238]. Most commercial cocktails are fungal-derived because several fungi are efficient degraders of plant biomass and show high production levels of catalytically efficient cellulases. Family GH7 cellulases are generally considered to be highly efficient and are only found in fungi. Fungi secrete lignocellulose-degrading enzymes into the medium, enabling easy separation from the producing organism Merino and Cherry [238]. However, fungal secretome profiles differ between fungal strains and may vary a lot depending on the carbon source [2, 30, 59, 240, 278]. This must be carefully considered when trying to select natural enzymes for conversion of differently pretreated biomass feedstocks. Despite a lack of publicly available data, it is clear that optimization of enzyme cocktails will have different outcomes for different feedstocks and that a one-size-fits-all strategy may not be optimal [33, 136].

Through the years, individual components of the enzyme cocktails have been the subject of enzyme improvement [268], either through screening for novel enzymes from alternative organisms (e.g., [133, 299, 326]) or by applying enzyme engineering technologies (e.g., [6, 80, 244, 313]). Work done on commercial enzymes is not generally known to the public; typical targets for improvement of individual cellulases include increased hydrolytic efficiency and/or stability at process conditions, reduced end-product inhibition and reduced lignin binding. Enzyme engineering strategies include directed evolution, usually based on combining random and site-directed mutagenesis steps [124, 244, 368], modification of the linker region of bimodular cellulases [14, 313] and domain shuffling, i.e., creation of fusion/chimeric proteins by combining (partial or complete sequences of) catalytic domains and CBMs from different enzymes/organisms [138, 331, 337, 369]. Despite the tremendous work that has been done for cellulase optimization, we are still trying to understand certain fundamentals of how EGs and CBHs work, and work together, the aim being to develop better (mixtures of) EGs and CBHs [176, 203, 257, 303, 362].

The significance of BG activity in alleviating end-product inhibition of CBHs by cellobiose accumulating during lignocellulose conversion was already clear in the late 1970s [325]. Sternberg et al. [325] showed that *Aspergillus* secretomes contain high levels of BG and can be used to compensate for the insufficient levels of BG activity in *Trichoderma* secretomes. In an early and quite unique study, Nieves et al. [254] assessed 13 commercial enzyme preparations from seven companies, including Novozymes' Celluclast 1.5L derived from *T. reesei*, for cellulolytic (i.e. filter paper) and β-D-glucosidase activities. The results of this study confirmed that the ratio of β-glucosidase-to-cellulase activity was two orders of magnitude higher in the *A. niger* preparations than in the *T. reesei* preparations. Novozymes' Celluclast 1.5L had the lowest BG titer of the tested *T. reesei* cocktails. A more recent report by Merino and Cherry [238] from Novozymes Inc. showed that engineering the production strain for Celluclast 1.5L to express a BG from *A. oryzae* led to significant improvement in both the conversion yield and rate of cellulose saccharification by the cellulase preparation. Notably, cellulase cocktails that were subsequently launched on the market, including Novozymes' Cellubrix or Cellic CTec series, have increased BG activity [48, 166] and do not require supplementation with BG for obtaining maximum saccharification efficiency, indicating that the production strains have been developed to express BGs at sufficiently high levels. Novozymes have recently discontinued the sales of their BG product Novozym 188, which has been commonly used to supplement Celluclast 1.5L.

While the oxidative mechanism of LPMOs was not uncovered until 2010 [351], it was already clear in 2007 that these proteins, at the time classified into the GH61 family, had the potential to improve hydrolysis yields by *T. reesei*-produced cellulase cocktails. Merino and Cherry [238] observed that addition of certain *T. terrestris*-produced GH61s at less than 5% of the total protein load in hydrolysis reactions with Celluclast 1.5L enabled reductions in the total enzyme loading by up to two times. Similarly to BGs, GH61s, today called LPMOs, have been incorporated in the Cellic CTec series [48, 62, 135, 160, 250]. Of note, while the contribution of LPMOs to the efficiency of today's cellulase cocktails is clear and important [49, 65, 146, 167, 248–250], optimizing this impact is not easy and requires careful consideration of reaction conditions [60], as discussed below.

Depending on the substrate pretreatment method, hemicellulases may also play a critical role in lignocellulose depolymerization. When working with substrates pretreated using neutral or alkaline conditions, hemicellulases may be of particular importance as these methods often leave hemicellulose fractions more or less intact Merino and Cherry [238]. It is well established that xylanase supplementation enhances cellulose conversion in biomass prepared by leading pretreatment methods, such as AFEX, ARP and dilute acid treatments, and that this effect is due to the removal of insoluble xylan, which limits cellulose accessibility [196]. Xylanases may also contribute by conversion of soluble xylo-oligosaccharides, which can inhibit cellulases [242, 283] to monomers. A study by Hu et al. on saccharification of steam-pretreated corn stover and poplar showed that, in addition to LPMOs, xylanases contribute to the efficiency of Cellic CTec2 [146]. As another example, the data sheet for Dupont's Accellerase Trio shows that this cellulase preparation is enriched in xylanases [94]. To cope with the variation of hemicellulose types and contents in a broad range of industrial biomasses, enzyme companies have developed hemicellulolytic preparations (e.g., Novozymes' Cellic HTec, DuPont's Accellerase XC, Genencor's Multifect Xylanase, Dyadic's FibreZyme, and AB Vista's Econase XT) that may be used to supplement base cellulolytic preparations (e.g., Novozymes' Cellic CTec or DuPont's Accellerase 1500). Notably, lignocellulosic ethanol plants primarily work with grasses, e.g., bagasse, corn stover and giant reed, which contain high amounts of xylans but lack glucomannan. With the exploration of other potential feedstocks, including hardwood and especially softwood biomass, which contain other types of hemicelluloses, further improvement of enzyme cocktails on this front is likely needed (see below).

Improvement of fungal strains for production of monocomponent enzymes and enzyme cocktails

As recently reviewed by Bischof et al. [36], *Trichoderma reesei* was discovered by researchers at the Natick Army Research Laboratories during World War II. Screening of 14,000 moulds isolated from rotting cellulose-based army equipment in the Solomon Islands for the ability to degrade crystalline cellulose resulted in the identification of the renowned ancestor of all current commercial *T. reesei* strains, designated as QM6a. Random mutagenesis of the *T. reesei* strain QM6a at Rutgers University led to the *T. reesei* strain RUT-C30, which is the prototype hyperproducer of cellulases and is commercially available [36, 271]. One of the key breakthroughs was truncation of the CRE1 transcription factor responsible for repressing the transcription of cellulase genes in the presence of glucose, which led to a substantial increase in cellulase production [236]. Decades of genetic engineering of *T. reesei* has resulted in detailed knowledge of regulators and transcription factors involved in enzyme expression, which again has contributed to the generation of novel cellulase hyperproducing mutants, as reviewed by Bischof et al. [36]. Alternative to genetic engineering of transcriptional regulators, other approaches to enhance expression levels of lignocellulose-active enzymes in *T. reesei* entail understanding the external conditions that

affect transcription and expression levels in fungal hosts [314], as well as promoter engineering, epigenetic engineering and metabolic engineering [92].

While *T. reesei* has played a vital role in the history of understanding and exploiting natural lignocellulose-degrading enzyme systems, other filamentous fungal species, including *Aspergillus* sp. [82], *Neurospora crassa* [93] and *Myceliophthora thermophila* [365], have also been studied in detail and may provide useful sources of enzymes or be developed as expression hosts for production of monocomponent enzymes or cellulase cocktails. Expression of recombinant proteins in filamentous fungi is traditionally based on the use of native expression systems, using innate transcriptional regulators and promoters. Transcriptional regulatory systems have been extensively studied in a wide variety of filamentous fungi [106, 241], and it has become clear that these systems are not widely conserved. Hence, knowledge of these systems is often not transferrable from one host organism to another, which is one of the reasons why the development of new filamentous fungal expression hosts is relatively slow [106, 241]. For species such as *T. reesei*, *A. niger* and *A. oryzae*, important regulatory systems are well-explored, as recently reviewed by Mojzita et al. [241]. In addition, relevant transcriptional regulators have been studied to varying extents for *N. crassa* [70, 197], *M. thermophila* [365, 378] and *Thermoascus aurantiacus* [309].

For the production of monocomponent enzymes, the target gene is commonly expressed under a strong promoter [22, 58, 106, 282, 365]. In some cases, rational engineering of the promoter may be used to enhance selective production of a recombinant protein in filamentous fungi; however, this approach is complex and often requires large-scale changes to entire gene networks [106]. Synthetic promoters are currently being considered more promising, since these can contribute to metabolism-independent protein expression [290]. Interestingly, external environmental factors such as light may affect the expression of plant cell wall-degrading proteins in filamentous fungi [308] and such factors thus need to be considered. A recent review on the use of light-regulated promoters addresses the potential of using external environmental factors to induce expression of heterologous proteins in filamentous fungi [118].

Additional strategies for improving fungal production of heterologous proteins include introducing multiple copies of the gene of interest into the expression host [390], fusing target genes to innate genes that are strongly transcribed and developing protease-deficient strains [75]. Most importantly, fungal strain development also includes the production of strains with low (hemi)cellulolytic background tailored for production of single enzymes or completely defined enzyme cocktails. Current industrial strains include Novozymes Inc.' protease-deficient *A. oryzae* JaL250 strain [390] as well as Roal Oy's cellulase-deficient *T. reesei* strain [329], DSM's cellulase-deficient *T. reesei* strain [5] and DSM's protease- and (hemi)cellulase-deficient *M. thermophila* (previously *Chrysosporium lucknowense*) LC strain [281, 365]. Of note, these strains are the results of major (commercial) research investments and are not publicly available.

Recent work by Steven Singer and co-workers has demonstrated that *T. aurantiacus* has a promising potential to become a thermophilic fungal expression host. *T. aurantiacus* secretes a limited number of endogenous plant cell wall-degrading enzymes, and the natural secretome, despite being relatively simple, has high efficiency in biomass hydrolysis [233, 309]. As a first step, the Singer team has shown that xylose acts as an inducer for production of both cellulases and xylanases in *T. aurantiacus* [310] and has identified related regulatory elements, homologues of which occur in the genomes of other Ascomycetes [309].

While traditional strain development of fungal strains is tedious and time-consuming, the availability of an ever-expanding number of fungal genome sequences through the Joint Genome Institute's 1000 Fungal Genomes Project [162] and advanced gene-editing technologies [289] together enable the development of alternative fungal enzyme factories. Novel CRISPR/Cas9-based tools will facilitate the development of a variety of novel fungal hosts for heterologous protein production. Indeed, CRISPR/Cas9 has already been adapted successfully to engineer cellulase hyperproducing strains of *Myceliophthora* species [217] and to recombinantly express enzymes in filamentous fungal hosts [290].

Identification of missing and underperforming enzyme components

Depending on the type of biomass and pretreatment technology, pretreated biomass feedstocks differ in composition and structure and thus hydrolysability by the same cellulase preparation, indicating the need for tailoring enzyme cocktails to the feedstock [143, 196, 318]. In addition to chemical composition and substrate structure, the soluble fraction of pretreated biomass, containing xylo-oligosaccharides and water-soluble lignin degradation products, may restrict the efficiency of some enzymes, due to inhibitory effects, while it may boost the efficiency of others, in particular LPMOs [226, 283, 381, 396]. Detailed studies have confirmed that the type of pretreatment impacts the efficiency of individual enzyme components, such as the CBH *Tr*Cel7A from *Hypocrea jecorina* (anamorph *T. reesei*) [159] and the LPMO *Ta*AA9A from *T. aurantiacus* [143], which, in turn, affects the optimal composition of the enzyme cocktail necessary for breaking down the feedstock [144]. Therefore, the use of industrially relevant pretreated substrates is a prerequisite when evaluating the efficiency of enzyme cocktails and

when trying to identify key enzyme activities that may be missing or underrepresented in the enzyme cocktail.

What have we learnt from minimal enzyme cocktail studies?

As a first approximation, optimizing the composition of a core set of cellulases, possibly also including one or more hemicellulases, for maximizing saccharification of pretreated feedstock gives good indications as to which enzyme components are important. In general, minimal enzyme cocktail studies have confirmed that there is no "one-fits-all" enzyme cocktail and that the ratio of enzyme components in the optimized mixture depends both on the type of biomass and pretreatment [21, 168, 174]. As an example, mannanases are not required for the saccharification of grasses, such as corn stover, which contain no glucomannan, while mannanase activity is essential for the saccharification of pretreated feedstocks that contain < 2% (even as low as 0.2%, w/w) glucomannan [21, 355]. In another study, Chylenski et al. showed that a four-component enzyme mixture that consists of *Tr*Cel7A and *Tr*Cel6A (CBHs), *Tr*Cel7B (EG) and *An*Cel3A (BG) and that had been optimized for degradation of sulfite-pretreated spruce was equally or more efficient than Cellic CTec2 and CTec3 [61]. Analysis of the hemicellulase activities of the optimized and commercial enzyme mixtures indicated that the efficiency of the minimal enzyme mixture on spruce most likely stems from its higher activity against glucomannan as compared with the commercial preparations. It is well known that *Tr*Cel7B can not only act on cellulose but also on glucomannan [173, 239].

Importantly, three independent studies have found that the proportion of the xylan-active EG *Tr*Cel7B (19–30%, w/w) is significantly more important than that of another EG, *Tr*Cel5A (0–2%, w/w), in enzyme mixtures optimized for saccharification of pretreated barley straw, corn stover and wheat straw [21, 168, 300]. When optimizing a 16-component *T. reesei* enzyme mixture for the saccharification of AFEX-treated corn stover, Banerjee et al. found that *Tr*Cel7A, *Tr*Cel7B, *Tr*Cel61A (=*Tr*AA9A), *Tr*Xyn11A, and *Tr*Xyn10A and the *Tr*Cel3A BG were the most important components [21], emphasizing the importance and complementarity of processive CBHs, promiscuous (i.e., xylan-active) EGs, LPMOs and xylanases for complete biomass degradation. Notably, only a handful of studies included LPMOs in their enzyme mixtures [21, 61, 87, 174]. The results of these studies indicate a correlation between the lignin content of the pretreated feedstock and the importance of LPMO in the enzyme mixture, which may be attributed to the ability of lignin to drive LPMO reaction, as discussed above (e.g., [185, 381]). When assessing the optimal proportion of LPMO in the enzyme mix, process conditions will have to be taken into account, too, since the LPMO reaction requires a source of oxygen.

While most minimal enzyme cocktail studies address interactions between the major *T. reesei* cellulases [21, 61, 168, 355], some have also looked at thermostable CBHs and EGs from alternative fungal species, such as *M. thermophila*, *T. aurantiacus* and *Chaetomium thermophilum* [87, 128, 168]. In processes run at higher temperatures, higher conversion yields can be achieved with (optimized mixtures of) thermostable enzymes as compared with *T. reesei* enzymes [168]. LPMOs from thermophilic fungi, such as *Ta*AA9A from *T. aurantiacus* [134, 146, 148, 272, 284] and AA9 LPMOs from *M. thermophila* [114, 117], have gained considerable interest recently. *Ta*AA9A, for example, is a good candidate for being added to cellulase cocktails [250]; however, there is no publicly available information on whether it has been incorporated into today's state-of-the-art commercial cellulase mixtures. Although thermostable enzymes have clear advantages in industrial settings, currently, no thermostable cellulase cocktails are available commercially [262].

Spiking studies to highlight enzyme activities lacking in commercial cellulase mixtures

Another, more direct approach to identify underperforming enzyme activities in cellulase cocktails is the supplementation or partial replacement of enzyme cocktails with either individual enzymes [134] or fungal broths [299]. An early example includes the supplementation of the *T. reesei*-derived Celluclast 1.5 cocktail with *A. oryzae*-produced Novozym 188 to compensate for the limited BG activity (e.g., in [299]). Analogously, several studies have shown co-operativity between commercially available cellulase, xylanase and pectinase preparations [19, 33, 119, 145], using combinations of products such as Accellerase 1000, Celluclast 1.5L, Spezyme CP, Multifect Xylanase, Multifect Pectinase and Viscozyme L. These studies add further proof to the general observation that no commercial cellulase preparation fits all substrates and highlight the importance of feedstock-specific enzyme blends.

To identify enzyme components that may be lacking in cellulase cocktails, commercial cellulase mixtures have also been supplemented with fungal culture broths or (semi)purified enzyme components. Celluclast has been studied extensively in spiking studies, revealing the positive impact of xylanase, mannanase and LPMO supplementation on the efficiency of cellulose saccharification [81, 87, 143, 177, 250, 272, 382], as also discussed above. In some cases, in-house fungal (e.g., *T. reesei*) culture broths have been used to showcase the positive effect of selected enzymes, such as three AA9s from *Geotrichum candidum* [205] or two AA14 LPMOs from *Pycnoporus coccineus* [68],

on saccharification efficiency. The direct effects of these (purified monocomponent) enzymes will also have to be tested on the latest generation (hemi)cellulase cocktails for benchmarking.

The most recent commercial cellulase cocktails have also been subjected to spiking-type of studies. As an example, Agrawal et al. have shown that the performance of Cellic CTec2 on acid or alkali pretreated bagasse and rice straw can be boosted by addition of two AA9 LPMOs from the thermophilic fungi *Scytalidium thermophilum* and *Malbranchea cinnamomea* [8]. Very recently, von Freiesleben et al. have reported that supplementation with GH5 and GH26 mannanases leads to improved saccharification of pretreated lodgepole pine by Cellic CTec3 [367], confirming previous indications concerning suboptimal levels of mannanase activities in Cellic CTec3 for softwood saccharification [61]. As another example, d'Errico et al. showed that a Cellic CTec preparation and the β-glucanase preparation UltraFlo possess only low amounts of glucuronoyl esterase activity and that supplementing these products with CE15 glucuronoyl esterases boosts their saccharification efficiency on pretreated corn fiber [77]. The positive effect of CE15 supplementation on the saccharification yields varied with the substrate [77], further corroborating the importance of feedstock-specific enzyme blends.

The interplay between process configuration and enzyme efficiency

The main considerations for process optimization entail (1) the type of feedstock and pretreatment method, (2) the choice of enzymes and their pH and temperature optima, (3) separate (SHF) or simultaneous (SSF) saccharification and fermentation steps, (4) stirring and aeration, (5) the possibility of on-site enzyme production and (6) possible measures for enzyme recycling. The choice of the process configuration (such as pretreatment, SHF/SSF and enzyme recycling) and physical parameters (such as temperature and level of dissolved oxygen) will have consequences for enzyme activity and stability. Of note, the enzymatic process is often separated into two phases: an initial liquefaction phase, in which the solid, particle-like feedstock becomes "fluid" (pumpable) and a saccharification phase, in which the polysaccharides are completely converted to soluble (mono-)sugars.

The choice of feedstock and pretreatment has a large impact on the type and amount of lignin remaining in the feedstock and, consequently, on the efficiency of both cellulases (in terms of the extent of unproductive binding) and LPMOs (in terms of delivery of reducing power). The temperature used during the enzymatic step(s) has to be carefully selected to compromise between enzyme efficiency and enzyme inactivation. Notably, the use of thermostable enzymes next to regular, less thermostable, cellulase cocktails will require alternative process configurations [363]. One possible scenario may be a liquefaction step run at elevated temperatures with a few selected thermostable enzymes, followed by full saccharification at lower temperature. In SSF, obviously, the temperature needs to be adapted to the fermenting microorganism. Of note, the impact of temperature goes beyond the impact on enzyme stability and activity, since temperature also affects potentially important abiotic factors such as reductant stability and dissolved oxygen levels, which may affect LPMO activity and/or the in situ generation of reactive oxygen species.

The improved efficiency of Cellic CTec2 compared to former, less efficient cellulase cocktails partly stems from the inclusion of LPMOs [146, 250]. The presence of molecular oxygen and/or H_2O_2 (Fig. 3) is crucial for LPMO activity, which will have to be considered in process design in general, and when choosing between SHF and SSF in particular. In a study comparing lactic acid production in different process setups, it was found that SHF performed better than SSF, and this was ascribed to the consumption of oxygen by the fermenting organisms in SSF, which lowered LPMO activity [249]. This is opposite to what has been observed in experiments with non-LPMO-containing cellulase cocktails, where SSF processes tend to be more efficient [49, 230, 256, 344]. Interestingly, Cannella and Jørgensen showed that the relative performance of SSF and SHF approaches varied with substrate loading [49]. At 20% (w/w) substrate loading of wheat straw, SSF with LPMO-containing Cellic CTec2 performed better, but at 30% (w/w) substrate loading the SHF approach yielded more ethanol, possibly because LPMO activity, which is only expected in the SHF approach, becomes more important at higher substrate concentrations [49]. With the possibility of direct supply of low (i.e., non-lethal) amounts of H_2O_2 to saccharification reactions, in particular for low-lignin feedstocks, a more efficient SSF setup that fully harnesses the power of LPMOs may become possible, since this would avoid competition for oxygen between the fermenting organism and in situ generation of H_2O_2. However, so far no studies have been published on this topic.

Overall process economics and efficiency may be increased further by producing enzymes on site, instead of using (combinations of) commercially available cellulase cocktails [161]. The carbon source used in growth media has been shown to have clear impacts on the protein expression profile of fungal expression strains [255]. Thus, on-site enzyme production may allow for tailoring the cellulase cocktail (i.e., the composition of the fungal secretome) to the feedstock of the biorefinery, by using this feedstock as the carbon source when cultivating the cellulase expression strain [1, 255].

Since enzymes are catalysts and, in principle, could be used many times, enzyme recycling may be considered during process design [147, 164]. Enzyme recycling

is a complex process that requires in-depth knowledge of enzyme–substrate interactions [346] and the mechanisms of enzyme adsorption–desorption [258, 280, 342, 358]. In principle, enzyme recycling could be done in two ways, either recycling the unhydrolyzed solid residue with bound enzymes or recycling the liquid phase with free (non-bound) enzymes [294, 295]. Both approaches have shown potential for saving enzyme costs [137, 293, 348], but they also make the process more complex. It is important to note that while enzyme recycling may seem attractive and "simple", such recycling has some intrinsic limitations. At the end of the hydrolysis, key enzyme components may be diluted out in the recycled enzyme fraction as different enzyme components will remain free or adsorbed on the feedstock as well as become inactive to various degrees [215, 280, 358]. LPMOs likely suffer from autocatalytic inactivation, especially when substrate concentrations become low in the later phase of a degradation reaction (see above), whereas it is well known that certain cellulases may get "stuck" by non-productive binding to cellulose in an essentially irreversible fashion [156, 232, 259, 267].

Importantly, one of the current targets when optimizing saccharification setups concerns how to leverage LPMO activity while keeping LPMOs from inactivation. As discussed above, LPMO inactivation may be caused by reactive oxygen species that derive from reactions between O_2 and lignin [185] or that are formed by the LPMO itself [37] or by other redox enzymes present in the enzyme mixture [39]. It has been shown for various reaction setups that too high feeding rates of externally added H_2O_2 [200, 248] or too high levels of in situ production of H_2O_2 [185, 269] lead to LPMO inactivation. Recent studies following the accumulation of LPMO products over the course of H_2O_2-assisted saccharification of industrial feedstocks [37, 65, 167, 248] clearly indicate that LPMO inactivation occurs presumably due to the accumulation of H_2O_2 in the reaction mixture, although the extent and rate of inactivation over time remain to be elucidated. Notably, there is a clear difference between LPMOs in terms of redox stability [66, 272], partly due to the presence or absence of CBMs (discussed below). Consequently, process robustness may be increased by screening for LPMOs with higher stability. Successful process optimization may further include control of the rate of addition or in situ generation of H_2O_2, control of dissolved oxygen levels, supplementation with catalase and/or superoxide dismutase to maintain low levels of H_2O_2 and superoxide radicals [37] as well as online monitoring and control of the redox processes taking place during saccharification, e.g., through online monitoring of the oxidation–reduction potential [167]. Before the power of LPMOs can be leveraged to its fullest extent, however, further fundamental research is required to better understand the impact of reactive oxygen species generated in biotic and abiotic redox processes on LPMO activity and to unravel the mechanisms of LPMO inactivation in the presence of industrially relevant feedstocks.

The role of CBMs: for cellulases, hemicellulases and LPMOs

Many of the enzymes discussed above contain one, or sometimes more than one, additional domain referred to as carbohydrate-binding module (CBM) [40]. Such modules may bind to various faces of cellulose crystals, to the more amorphous regions of cellulose or to one or more hemicellulose types [51, 234]. Accordingly, some CBMs target surfaces (i.e., multiple polysaccharide chains, such as the CBM1 of *Tr*Cel7A), others target single polysaccharide chains, whereas the third type directs the catalytic domain to act at polysaccharide chain ends [123]. Substrate-binding by CBMs, while being fully reversible [90, 216, 267], may be very strong, because of which it has sometimes even been considered almost irreversible [52, 292]. Irreversible binding would be puzzling since it does not seem favorable for enzyme efficiency. There have been many theories about what CBMs do and how they work, including proposals that some CBMs may increase substrate accessibility by disrupting the crystalline structure of cellulose [40, 127]. The primary role of CBMs, with massive experimental support, is that they promote proximity between the appended catalytic domain and the substrate, thus promoting enzyme efficiency.

To some extent, CBMs and substrate binding are a double-edged sword in saccharification efficiency. On the one hand, CBMs increase the enzyme's affinity to its substrate [184], which promotes enzyme activity on insoluble cellulose [345, 353]. For processive CBHs, the CBM has been proposed to promote the feeding of the cellulose chain into the CBH active site [184] and to increase processivity [25, 153, 181, 333], as well as to promote the stability of the CBH-cellulose complex. On the other hand, strong substrate binding via CBMs hinders desorption of bound enzymes [74, 333], which may get stuck on the substrate [199]. Moreover, CBMs contribute to unproductive binding of cellulases to lignin [286, 287, 321], which may result in enzyme inactivation.

The proximity effect of CBMs can be compensated by increasing substrate concentration, which will promote substrate binding of enzymes independent of the presence of a CBM. In 2013, Várnai et al. showed that, at high substrate concentrations, the truncated, CBM-free versions of the four CBM-containing cellulases from *T. reesei* (*Tr*Cel7A, 6A, 7B and 5A) were as efficient as the full-length enzymes [356]. Since then, the positive effect of increasing substrate concentration on the efficiency of cellulases and LPMOs without CBMs has been confirmed by a number of studies, as has the potentially negative impact of CBMs in reactions with high

substrate concentrations [53, 66, 158, 170, 210, 334]. This observation can be explained by CBM-free cellulases having higher desorption rates ("off-rates") [333] and reduced unproductive binding to lignin [260, 288], while increased substrate concentrations will overcome diffusional limitations of the CBM-free enzymes [372]. Of note, the presence or absence of CBMs in the enzyme components will affect potential enzyme recycling strategies. Using CBM-free enzymes will facilitate recycling unbound enzymes from the liquid phase [137, 258], while CBM-containing enzymes may be recycled in a bound form, with the unhydrolyzed solid residue and/or after desorption from the unhydrolyzed solid residue [211, 294, 295, 348].

CBMs also occur in LPMOs, although many LPMOs, including some of the best-studied ones with documented effects on cellulose saccharification [143, 178, 250, 284], lack CBMs. LPMO literature shows that certain single-domain LPMOs bind very well to their substrates, whereas recombinantly expressed catalytic domains of CBM-containing LPMOs sometimes seem to bind weakly [66, 110, 132]. It may thus seem that nature has evolved different strategies for LPMOs to have affinity for their substrates, but this is not yet sufficiently supported by systematic experimental studies. Existing data show that the CBMs of LPMOs have the same function as in GHs [53, 66, 72, 110, 111, 192, 209, 371] and it has also been shown that, like for GHs, the presence of a CBM becomes less important, and even unfavorable, when running reactions at high substrate concentrations [66].

Importantly, LPMOs that are reduced and meet O_2 or H_2O_2 while not being bound to the substrate are prone to autocatalytic inactivation, due to the redox reactivity of the Cu(I) ion in the (reduced) catalytic center [37]. Thus, for LPMOs, proximity of the substrate not only promotes activity, but also stability, since proximity of the substrate increases the chances for the LPMO to engage in productive (i.e., oxidative cleavage of the substrate) rather than damaging side reactions. Several studies have shown that deletion of the CBM from a CBM-containing LPMO indeed leads to increased enzyme inactivation [66, 108, 273]. On the other hand, LPMOs have been found to bind more strongly to polysaccharides when the active site copper is in the reduced, i.e., Cu(I), state [188, 201], which is expected to favor their stability.

Interestingly, the importance of the proximity effect was also suggested by experiments with a cellulose-binding CBM-containing pyrroloquinoline quinone-dependent pyranose dehydrogenase (PDH) that can deliver reducing equivalents to LPMOs and thus drive the LPMO reaction. Upon removal of the CBM from this PDH, the LPMO reaction became less efficient and it has been suggested that this is due to proximity effects [357]. When the PDH is bound to cellulose, it will activate the LPMO while the LPMO is in close proximity to the substrate. On the other hand, a PDH that is free in solution will activate LPMOs that are not close to the substrate, thus increasing the chances for off-pathway reactions.

Concluding remarks

Thanks to the efforts of a large research community and enzyme companies, today's enzyme cocktails for saccharification of lignocellulosic biomass are so effective that industrial bioethanol production from such biomass has become a reality. Improved biomass pretreatment techniques have contributed to this development [391]. Despite much progress in the enzyme area, further improvements still seem possible. For example, it is still not fully clear how processive cellulases work and how the interplay of these essential but rather slow enzymes with other enzymes could be optimized [57, 169, 302, 306, 362]. Recent insights concerning the role of H_2O_2 and enzyme inactivation suggest that so far, we have not harnessed the full potential of LPMOs. Furthermore, despite much research on LPMOs in the past decade, exactly how these enzymes co-operate with classical cellulases remains largely unknown (see [343] for a recent study). Finally, recent work suggests that LPMOs could play a role in removing (traces of) recalcitrant hemicellulose, which may promote cellulolytic processes [68, 150]. On that note, further research on the impact of residual hemicellulose fractions in pretreated biomass and the possible roles of (any) hemicellulolytic enzymes in dealing with such fractions is still needed.

While research related to the enzymatic processing of lignocellulosic biomass has focused mainly on conversion of the polysaccharides, there is growing evidence that biomass saccharification and lignin modification by enzymes are interconnected [39]. Although our current understanding of enzymatic processing of lignin is still very limited, there is a growing interest in lignin valorization. As lignin constitutes nearly a third of plant biomass, the fate of the lignin fraction will need to be considered in the further development of biorefining processes for efficient and economic processing of lignocellulosic feedstocks [24, 285, 311]. A good example for the way forward is the so-called BALI process, where sulfite pretreatment generates both valuable carbohydrate and lignin streams which can be turned into valuable products [65, 301].

In addition to lignin valorization, there is a concerted ongoing research effort aimed at developing a widened portfolio of biomass-derived products, including cellulose-, hemicellulose-, and lignin-based polymers, oligomers and monomers, as well as products resulting from fermentation of lignocellulosic sugars, i.e., production of ethanol. Alternative fermentation products include microbial biomass for

food and feed [29, 207, 336], alternative biofuels such as butanol [237] and commodity as well as high-value chemicals [317, 384]. In an environmentally and economically successful biorefinery, these products will co-exist as part of a flexible product portfolio that is continuously adjusted to feedstock availability, technological developments and market needs.

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Paper 2

2-Naphthol impregnation prior to steam explosion promotes LPMO-assisted enzymatic saccharification of spruce and yields high-purity lignin

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Research Article

2-Naphthol Impregnation Prior to Steam Explosion Promotes LPMO-Assisted Enzymatic Saccharification of Spruce and Yields High-Purity Lignin

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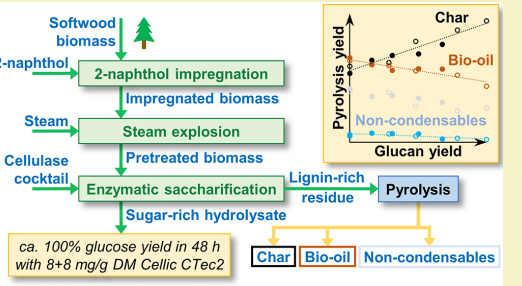
ABSTRACT: The recent discovery that impregnation with a carbocation scavenger may improve the enzymatic saccharification of steam-exploded softwood has brought a softwood-based biorefinery closer to reality. However, the nature of the impregnation effect remains unresolved, and its impact on process efficiency and product quality in high-dry matter reactions remains underexplored. Here, we show that 2-naphthol impregnation enables the complete saccharification of spruce cellulose by lytic polysaccharide monooxygenase (LPMO)-containing Cellic CTec2, but not by an LPMO-poor cellulase cocktail (Celluclast), in 10% dry matter reactions with an industrially feasible enzyme dose and reaction time. Importantly, we show that this remarkably high saccharification yield correlates with increased LPMO activity, which is due to the impact of 2-naphthol on the ability of lignin to drive the LPMO reaction. These findings show that impregnation improves saccharification not only by reducing cellulase adsorption and inactivation but also by boosting oxidative cellulose depolymerization by LPMOs. Pyrolysis of the lignin-rich saccharification residues revealed that 2-naphthol impregnation had little effect on lignin-derived components in the resulting bio-oil, which, due to the efficient saccharification, showed reduced levels of carbohydrate-derived components that reduce oil storage stability. These results bring closer the prospect of a spruce-based biorefinery that combines biochemical and thermochemical conversion routes.



KEYWORDS: *steam explosion, carbocation scavengers, enzymatic saccharification, LPMO, lignin, pyrolysis oil, biorefinery*

INTRODUCTION

Norway spruce, a softwood, is an abundant natural resource that can be found in Canada, the Nordic countries, and Russia and, as such, is a good starting material for the generation of biobased products, such as biofuels, biochemicals, and biomaterials, in a wood-based biorefinery. One key process in such a biorefinery would be the enzymatic saccharification of cellulose to glucose followed by fermentative valorization to, for example, bioethanol[1] or single-cell protein.[2] Currently, hardwoods, such as birch, and agricultural crops, like sugarcane bagasse and corn stover, are the main feedstocks used in efforts to commercialize second-generation bioethanol production.[3,4] With the exceptions of St1's Cellunolix and Borregaard's ChemCell ethanol projects in Northern Europe, there is a lack of softwood-based biorefineries, partly due to the high recalcitrance of softwood biomass.

In general, lignocellulosic biomass requires pretreatment prior to enzymatic saccharification to render the plant cell wall more accessible to the enzymes. Steam pretreatment is one of the most common pretreatment technologies used in lignocellulosic biorefineries[5] and is applicable to softwood, especially when combined with explosive decompression[6] and an acid catalyst.[7,8] While efficient in removing hemicellulose and increasing cellulose accessibility, the severe conditions of steam explosion lead to the condensation of lignin fragments,[9] especially in guaiacyl-rich softwood lignin.[10,11] The resulting lignin fraction will inhibit subsequent enzymatic cellulose depolymerization due to effects on unproductive enzyme adsorption[12,13] or because reprecipitated lignin may sterically hinder cellulose accessibility.[14,15] Notably, carbocation scavengers such as 2-naphthol have been found to prevent/quench the coupling of oligomeric lignin fractions and, therefore, lignin

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condensation[9,10,16] as well as improve the enzymatic digestibility of cellulose in steam-pretreated feedstocks.[11]

In a biorefinery setup based on biochemical conversion processes, the lignin-rich residue that is obtained after enzymatic saccharification is most commonly burnt to produce steam and power. Alternatively, it could be upgraded to blends of liquid, gaseous, or solid fuels via thermochemical conversion processes such as pyrolysis or hydrothermal liquefaction, as shown by recent examples.[17,18] When using steam explosion as pretreatment, 2-naphthol impregnation is more advantageous compared to SO_2 impregnation as it results in a more reactive and sulfur-free lignin[10,19] suitable for the production of higher-value products, like polymers or advanced fuels. Considering sustainability, the use of 2-naphthol violates principles of green chemistry because it is a toxic compound[20] derived from crude oil. Importantly, more eco-friendly but hitherto less effective alternatives exist, such as mannitol from seaweed[21] and lignin-derived phenolic acids.[21,22] On the other hand, 2-naphthol may be obtained from renewable resources, such as lignin itself, e.g., via thermochemical processing at low oxygen levels.[20,23] Here, we used 2-naphthol as a model compound to study the impact of impregnation with a carbocation scavenger on a process setup combining biochemical and thermochemical conversion of spruce wood.

While inhibitory effects of lignin on saccharification efficiency are well established,[24] it has recently been shown that lignin may promote cellulose degradation because certain lignin fractions promote the activity of lytic polysaccharide monooxygenases (LPMOs).[25−28] LPMOs are key components of today's state-of-the-art cellulase cocktails[24,29] and need electrons and O_2 or H_2O_2 to function.[30−32] It has been shown that lignin can provide LPMOs with electrons and that reactions between lignin and O_2 generate H_2O_2, which speeds up the LPMO reaction.[28,33]

As 2-naphthol impregnation during steam explosion has been proposed to affect lignin reactivity,[10] we set out to investigate the impact of 2-naphthol impregnation on LPMO activity and the overall saccharification efficiency of a commercial LPMO-containing cellulase cocktail, Cellic CTec2. We show that 2-naphthol drastically enhances the saccharification of pretreated spruce at high dry matter (10% w/w), to the extent that close to 100% glycan conversion may be reached even for a feedstock as recalcitrant as spruce wood. Most importantly, based on the analysis of LPMO-generated reaction products and assessment of lignin reactivity, we show that the positive impact of 2-naphthol impregnation correlates with improved lignin-driven LPMO activity. Finally, we show that 2-naphthol impregnation improves the quality of bio-oils obtained upon thermochemical conversion of saccharification residues.

EXPERIMENTAL SECTION

Substrate Preparation. The feedstock used was the stem wood of debarked and drum-dried Norwegian spruce chips (3−5 mm) harvested in the Viken area in Norway. Spruce chips were milled to 1 mm particle sizes using a knife mill (SM2000, Retsch, Haan, Germany) equipped with a 1 mm sieve in 30 min intervals to prevent excess heat. After milling, 1750 g of milled spruce (dry matter, 95%) was impregnated with 0.205 M 2-naphthol (purity, 99%; Sigma-Aldrich St. Louis, USA) dissolved in acetone (41.1 g 2-naphthol in total), and the solvent was completely evaporated for 12 h.

Milled spruce, with and without 2-naphthol impregnation, was pretreated using steam explosion as described previously[34] using a steam explosion unit produced by Cambi A/S (Asker, Norway). Twelve batches of 350 g each (air-dried weight) were treated at 190, 200, 210, or 220 °C with a residence time of 5 or 10 min (see Table S1). All pretreated samples were stored at 4 °C.

The severity factor (R_0) was calculated using the following equation: $R_0 = t \cdot e^{T-100/14.75}$, where t is the residence time in min and T is the temperature in °C.[35] The dry matter (DM) content of all samples was determined by drying the feedstock overnight at 105 °C. Pretreated spruce feedstocks were analyzed for cellulose, hemicellulose, and lignin content based on the standard operating procedure developed by NREL.[36] Monomeric sugars were quantified with high-performance anion exchange chromatography (HPAEC) as described below.

Enzymatic Saccharification. For the saccharification trials, Cellic CTec2, Celluclast 1.5 L, and β-glucosidase were kindly provided by Novozymes A/S (Bagsværd, Denmark). The protein concentration was determined using the Bradford method with bovine serum albumin as the standard.[37] Celluclast (C) and β-glucosidase (BG) were mixed in a 9:1 ratio (on a protein basis) to overcome β-glucosidase deficiency in Celluclast while maximizing the cellulase activity of the Celluclast−BG mixture.

Enzymatic saccharification was carried out in 50 mL glass bottles sealed with rubber caps (Wheaton, Millville, USA) as described previously.[27] Reactions were set up in a working volume of 20 mL, with 10% DM (w/v) substrate and 50 mM (final concentration) sodium acetate buffer at pH 5.0. Prior to enzyme addition, the pH was adjusted with 1 M NaOH to set the final pH to 5.0, and the reactions were preincubated at 50 °C for 15 min. Enzymes (2, 4, or 8 mg/g DM enzyme loading) were added through the septa of the caps at t = 0 h to start the reaction; in some reactions, an additional dose of enzymes (8 mg/g DM) was added at t = 24 h. Reactions were incubated at 50 °C for 48 h with 200 rpm orbital shaking. All reactions, including controls without enzymes, were carried out in triplicates. For anaerobic reactions, the solutions were flushed with N_2 for 2 min at a flow rate of 300 mL/min prior to preincubation. For reactions with H_2O_2 additions, 20 μL of a 90 mM H_2O_2 solution was added every 60 min through the septa using a 50 μL Hamilton syringe to achieve a final H_2O_2 concentration of 90 μM, corresponding to a feed rate of 90 μM/h.

Whole slurry samples (300 μL each) were withdrawn through the septa using a wide-tip needle (2.10 mm × 80 mm) mounted to a 1 mL syringe, boiled immediately for 15 min in a heat block (Dry Block Heater 1, IKA), cooled on ice for 5 min, diluted 2−10-fold (to minimize overestimation of the saccharification yields[38]), and centrifuged at 20,000g without cooling for 2 min. The supernatants were filtered through a 0.45 μm hydrophilic filter using a 96-well filter plate (Millipore), operated with a vacuum manifold, and stored at −20 °C until further analysis.

Analysis of Monosaccharides and C4-Oxidized Products. Glucose in saccharification samples was analyzed by high-performance liquid chromatography (HPLC) using a Dionex Ultimate 3000 (Dionex, Sunnyvale, USA) connected to a refractive index detector 101 (Shodex, Japan) as described previously.[27] The column was a Rezex ROA-organic acid H+ (8%) 300 mm × 7.8 mm analytical column (Phenomenex, Torrance, CA, USA) kept at 65 °C, the eluent was 5 mM H_2SO_4, and the flow rate was 0.6 mL/min.

Monosaccharides (L-arabinose, D-galactose, D-glucose, D-xylose, and D-mannose) obtained during compositional analysis of pretreated feedstocks and the C4-oxidized dimer, Glc4gemGlc, found in enzymatic saccharification samples, were analyzed using a Dionex ICS-3000 (Dionex, Sunnyvale, USA) equipped with a CarboPac PA1 column (2 mm × 250 mm) and guard column (2 mm × 50 mm) kept at 30 °C and connected to a pulsed amperometric detector (PAD) as described previously.[17,27] Glc4gemGlc standards were produced as described by Müller et al.[27]

Evaluating the Capability of Pretreated Spruce to Drive LPMO Action. Pretreated feedstocks were used as reducing agents in reactions with a chitin-active LPMO, CBP21 (*Sm*AA10A) from *Serratia marcescens*.[30,39] Reactions (with 350 μL total volume) contained 1% (w/v) steam-exploded spruce, 1% (w/v) β-chitin from a squid pen with an average particle size of 0.8 mm (batch no.

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20140101; produced by France Chitine, Orange, France), and 1 μM CBP21 in 50 mM Bis−Tris/HCl buffer (pH 6.5). Reactions were set up in 2 mL Eppendorf tubes and incubated at 40 °C and 1000 rpm in an Eppendorf ThermoMixer (Eppendorf, Hamburg, Germany). Samples (50 μL) were withdrawn periodically from the reaction mixtures, mixed with 50 μL distilled water, and filtered immediately through a 96-well filter plate (Millipore) operated with a vacuum manifold to stop the reaction. Subsequently, all samples were treated with the chitobiase *Sm*GH20A from *S. marcescens* (UniProt ID Q54468) overnight at 37 °C to convert the LPMO products to a mixture of *N*-acetylglucosamine (GlcNAc) and chitobionic acid as described before.[40] GlcNAc and chitobionic acid were quantified using a Dionex Ultimate 3000 UHPLC system (Dionex, CA, USA) equipped with a Rezex RFQ-Fast acid H+ (8%) 100 mm × 7.8 mm column (Phenomenex, CA, USA) and UV detection with a previously established method.[41] For quantification, chitobionic acid standards were produced in-house,[40,42] and *N*-acetylglucosamine was purchased from Sigma-Aldrich (MO, USA).

Solid-State Nuclear Magnetic Resonance Spectroscopy of the Spruce Samples. SSNMR ^{1}H-^{13}C cross-polarization spectra were collected using a Bruker Avance III spectrometer operating at a magnetic field of 11.74 T. A 4.0 mm double resonance magic angle spinning probe head was used at room temperature with a magic angle spin rate of 12 kHz. The spectra were acquired using 12,000 scans, a recycle delay of 5 s, and a Hartmann−Hahn contact time of 2000 μs. Before Fourier transformation of the averaged signals/free induction decays, zero filling and apodization were applied to improve the line shape definitions and the signal-to-noise ratio. The apodization was done by multiplying the free induction decays with a decaying exponential window function with a processing line broadening factor of 150 Hz. All NMR spectra were then adjusted by proper signal phasing and baseline corrections. The chemical shifts were referenced to tetramethylsilane by the substitution method,[43] setting the high frequency peak of adamantane to 38.48 ppm. Cellulose and lignin peaks were identified according to the annotations by Wang et al.[44]

Fixed-Bed Pyrolysis. Native spruce (without (U) or with (N) 2-naphthol impregnation), steam-exploded spruce (without (U-220/10) or with (N-220/10) 2-naphthol impregnation), and saccharification residues of steam-exploded spruce treated with various amounts of Cellic CTec2 were dried at 60 °C under vacuum for 12 h to remove water before being subjected to pyrolysis using a fixed-bed pyrolysis unit as described in Kalyani et al.[17] The biomass samples (0.5 g) were sieved to a particle size of 200−500 μm, placed in a stainless-steel tube (sample tube), and kept in place using quartz wool. A constant nitrogen flow of 100 mL/min was applied to pass through the sample from the top, moving the produced pyrolysis vapors out of the high-temperature zone and leaving the nonvolatile biochar fraction behind in the sample tube. The oven was preheated separately, and once the desired temperature of 500 °C stabilized, the preheated oven was rapidly clamped around the sample tube containing the biomass samples. After a pyrolysis time of 15 min, the oven was removed from the sample tube. During the pyrolysis, the pyrolysis vapors leaving the sample tube were guided through a cooling trap with a temperature of −20 °C and cooled to separate the condensed liquid (a mixture of bio-oil and aqueous phase) and gaseous fractions.

Quantification and Compositional Analysis of Pyrolysis Products. After measuring the mass of the condensed liquid fractions, the liquids were diluted using tetrahydrofuran and spiked with 1% (w/w) decane as external standard. The water content was analyzed by the Karl Fischer method. The organic fraction of the condensed liquid fractions (i.e., bio-oil) was analyzed with gas chromatography (GC) coupled with mass spectrometry (MS) using a gas chromatograph connected with a mass spectrometer and a flame ionization detector (FID) on an Agilent GC × GC−MS/FID system. The MS detector was applied to identify the compounds, and the identified compounds were quantified by FID using the FID response factors described by de Saint Laumer et al.[45] The FID signals of the identified compounds accounted for more than 98% of the total sum of signals detected. The char yield was calculated as the weight ratio of the produced biochar and the dried biomass before pyrolysis, determined by weighing the sample tube three times: (1) empty (containing only quartz wool), (2) containing the dried biomass samples before pyrolysis, and (3) containing the produced biochar after pyrolysis. The temperature profile within the reactor was adjusted to ensure that the condensation of vapors occurred predominantly in the quartz sampling vial located below the pyrolysis tube. Only small amounts of heavy tar compounds condensed at the reactor tube outlet. Those were regarded as char-like products in the yield calculations. The mass of the noncondensable (gaseous) fraction was quantified from the mass difference of the pyrolysis feedstock (i.e., the dried biomass) and the sum of the condensed liquid fraction and biochar.

RESULTS AND DISCUSSION

Impact of 2-Naphthol Impregnation and Pretreatment Parameters on Feedstock Composition. Recently, Pielhop et al. have reported that 2-naphthol impregnation of spruce prior to steam pretreatment enhances subsequent enzymatic saccharification efficiency for pretreatments performed at severity levels ($\log R_0$) of 4.4 and higher.[46] To further explore the impact of 2-naphthol impregnation on the pretreatment of spruce at lower severity levels, we subjected batches of milled spruce with or without 2-naphthol impregnation (denoted by N and U, respectively) prior to steam explosion at severity levels of 3.65−4.53 (see Table S1). Regarding feedstock composition, the lignin content in the pretreated feedstock increased due to pseudo-lignin formation (as expected[47]), while the hemicellulose content (both xylan and glucomannan) decreased with increasing pretreatment severity (Table S2). Impregnation with 2-naphthol had little effect on the overall composition of the biomass after pretreatment (Table S2).

While 2-naphthol impregnation did not affect the apparent lignin content of the pretreated materials, SSNMR analyses showed effects of both the steam explosion as such and 2-naphthol impregnation on lignin structure but not on the cellulose structure, as detailed in the Supporting Information and Figures S1−S4. On the one hand, steam explosion (without 2-naphthol impregnation) led to depolymerization/cracking of lignin components as indicated by a relative increase in aromatic C atoms without substituents and a loss of methyl and methoxy groups. On the other hand, the presence of 2-naphthol during steam explosion led to a higher relative intensity of O-linked and nonsubstituted aromatic C atoms (compare U-220/10 and N-220/10 in Figure S3) or, in other words, to the retention of phenolic OH groups. Notably, some of the (nonsubstituted) aromatic C atoms may originate from the naphthalene ring of 2-naphthol in N-220/10.[11] Previously reported 2D-NMR analyses of pretreated aspen[10] and spruce[11] feedstocks showed that impregnation with 2-naphthol prior to steam explosion leads to a pretreated material where naphthalene rings are incorporated in the lignin while phenolic hydroxyl groups are retained, both indicating a reduced degree of lignin condensation. Our data are in accordance with these findings.

Impact of 2-Naphthol Impregnation on Enzymatic Digestibility of Spruce. Initial screening of all pretreated feedstocks (Table S1) for saccharification efficiency with the LPMO-containing cellulase cocktail Cellic CTec2 showed improved saccharification yields and a positive effect of 2-naphthol impregnation only for the more severe ($\log R_0 > 4.0$) pretreatment conditions (Figure S5). Thus, we selected the

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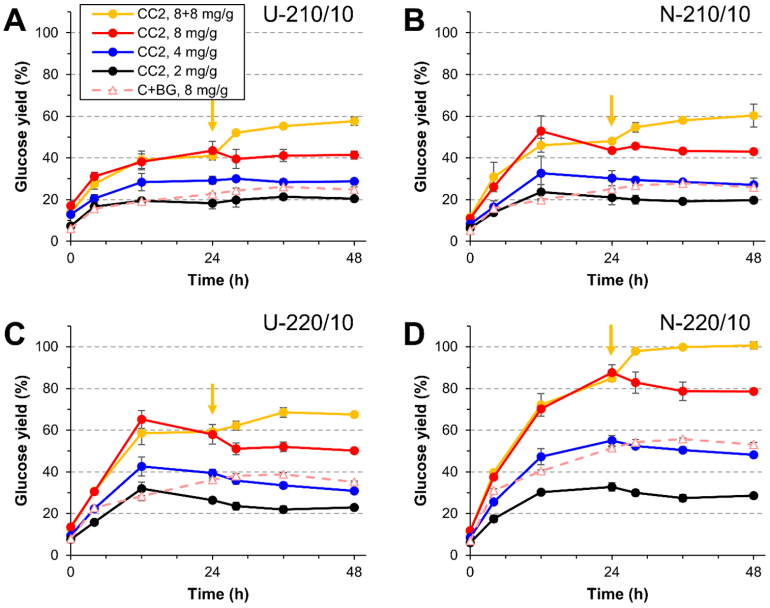

Figure 1. Glucose yields during enzymatic saccharification of pretreated Norway spruce. Norway spruce pretreated at 210 °C (A, B) or 220 °C (C, D) without impregnation (A, C) and with 2-naphthol impregnation (B, D) was subjected to saccharification with Cellic CTec2 (CC2) at various enzyme loadings or a Celluclast−β-glucosidase mixture (C + BG). In the reactions, 10% DM (w/v) substrate was incubated with 2 mg (black), 4 mg (blue), or 8 mg (red) of Cellic CTec2 or 8 mg of C + BG (light red, dashed line) protein per g of DM in 50 mM sodium acetate buffer (pH 5.0) at 50 °C. In the reaction marked as "8 + 8 mg/g" (yellow), 8 mg/g DM Cellic CTec2 was added at t = 0 h, and an additional 8 mg/g DM Cellic CTec2 was added at t = 24 h (marked with yellow arrows). Glucose yields are expressed as the percent of total glucan content. The error bars represent the standard deviation for the averages of three independent experiments.

four feedstocks pretreated at the highest severities (U-210/10, N-210/10, U-220/10, and N-220/10) for further experiments. Monitoring glucose release with Cellic CTec2 over time, at three levels of enzyme loading, revealed that 2-naphthol impregnation had only a small effect on biomass digestibility when the pretreatment was carried out at 210 °C ($\log R_0$ = 4.24; Figure 1A,B). Glucose yields obtained with 8 mg/g Cellic CTec2 loading could be increased by supplying additional enzymes after 24 h, showing that the enzyme cocktail was a limiting factor in these conditions. Pretreatment at 220 °C resulted in higher glucose yields, and in this case, 2-naphthol impregnation had a strong positive effect (Figure 1C,D). At all enzyme loadings, the glucose conversion yields after 24−48 h were 24−62% higher for N-220/10 than for U-220/10 (Figure 1C,D), and the impact of 2-naphthol impregnation was most pronounced in the reactions with the higher enzyme dose. Of note, a slight decreasing trend in glucose yields was visible after 12−24 h of saccharification for some reactions in Figure 1. The reason could be that sampling with a syringe at low saccharification levels can be challenging due to relatively large feedstock particles. This may have resulted in a lower DM content in some of the samples compared to that in the reaction slurry. Since the samples were diluted prior to centrifugation, this may have resulted in slight overestimation of glucose concentration in (some of the) early sample points. However, this issue did not affect the overall trends in Figure 1.

It is noteworthy that the high saccharification yields with N-220/10 (Figure 1D) were obtained using process parameters that are close to being industrially realistic (10%, w/v DM; 8−16 mg/g DM Cellic CTec2; 48 h incubation time). Earlier studies showing the impact of 2-naphthol were done using less realistic conditions. For example, in their pioneering work, Pielhop et al. used low substrate concentrations (1%, w/w), high enzyme dosages (Accellerase 1500 at 15−60 FPU/g cellulose, corresponding to ca. 12−48 mg protein/g DM[48]), and extended incubation times (120 h).[46] In previous studies,[19,21,22,46] the observed 2-naphthol effect on enzymatic saccharification was attributed to increased cellulose accessibility and reduced nonproductive binding of cellulases by lignin. Since lignin has been shown to drive LPMO reactions,[25,28] we considered whether the impact of 2-naphthol could relate to altered lignin properties and improved LPMO activity.

To assess possible LPMO-related effects, we set up saccharification reactions with Celluclast−BG, an LPMO-poor cellulase cocktail, at 8 mg/g enzyme loading level. As also reported before,[27,32] the reactions with the Celluclast−BG mixture generally gave lower saccharification yields compared to the corresponding reactions with Cellic CTec2, a next-generation, hence more powerful, enzyme cocktail[24,32] (Figure 1). Importantly, 2-naphthol impregnation with pretreatment at 220 °C enhanced saccharification with the Celluclast−BG mixture to a lesser extent (by 42−51%) compared to the reactions with Cellic CTec2 (by 51−62%, after 24−48 h incubation at 8 mg/g enzyme loading). The more prominent boosting effect observed for Cellic CTec2 especially at higher saccharification levels (reaching glucose yields of 79−88% with Cellic CTec2 vs 51−56% with the Celluclast−BG mixture for

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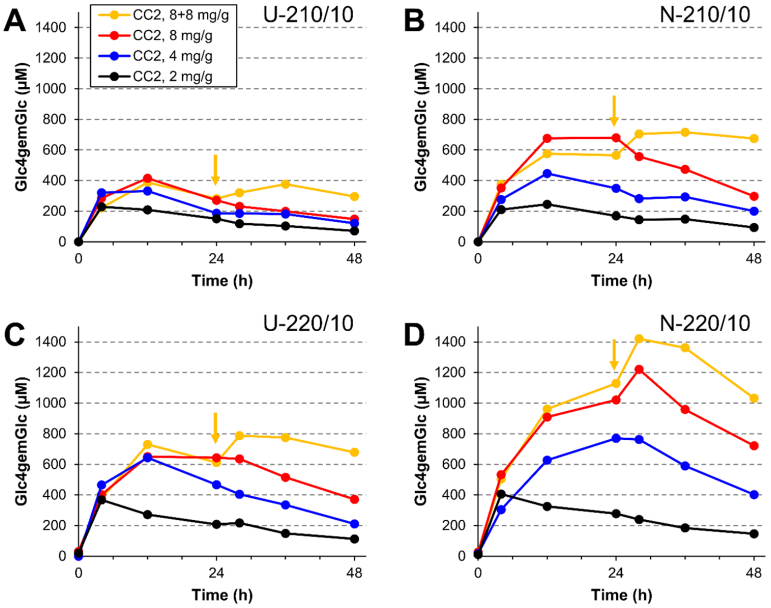

Figure 2. Glc4gemGlc (C4-oxidized cellobiose) levels during enzymatic saccharification of pretreated Norway spruce. Norway spruce pretreated at 210 °C (A, B) or 220 °C (C, D) without impregnation (A, C) and with 2-naphthol impregnation (B, D) was subjected to saccharification with Cellic CTec2 at various enzyme loadings. In the reactions, 10% DM (w/v) substrate was incubated with 2 mg/g (black), 4 mg/g (blue), or 8 mg/g (red) DM Cellic CTec2 (CC2) in 50 mM sodium acetate buffer (pH 5.0) at 50 °C. In the reaction marked as "8 + 8 mg/g" (yellow), 8 mg/g DM Cellic CTec2 was added at t = 0 h, and an additional 8 mg/g DM Cellic CTec2 was added at t = 24 h (marked with yellow arrows). The graphs show the Glc4gemGlc values measured for one of the three parallel reactions shown in Figure 1; another set of parallel reactions is provided in Figure S6, showing the same trends. In reactions with the 8 mg/g DM Celluclast−β-glucosidase mixture, no oxidized products were detected (not shown).

N-220/10) strongly suggests that the impact of 2-naphthol is related to specific enzymes in the enzyme cocktails, for example, LPMOs (see below). On the other hand, the fact that 2-naphthol impregnation at 220 °C improved saccharification efficiency also with the LPMO-poor Celluclast−BG mixture corroborates the impact of other factors like nonproductive binding of cellulases to lignin.

Impact of 2-Naphthol Impregnation on LPMO Activity. To assess the impact of 2-naphthol impregnation on LPMO activity, we quantified the main LPMO product (i.e., C4-oxidized cellobiose, Glc4gemGlc[27]) in the reactions shown in Figure 1. The dominant LPMO in Cellic CTec2 generates C4-oxidized oligosaccharides, which are depolymerized to the dimer Glc4gemGlc by endoglucanases. The oxidation at the C4 position of the sugar at the nonreducing end hinders further depolymerization to monomers by β-glucosidases.[27] In general, 2-naphthol impregnation led to increased levels of soluble LPMO products in reactions with Cellic CTec2 (Figure 2). In the reactions with the LPMO-poor Celluclast−BG mixture, no oxidized products were detected. As observed for glucose release (Figure 1), the positive impact of 2-naphthol on the Glc4gemGlc production was most prominent when using the highest pretreatment temperature and the highest enzyme dosage. The curve shapes indicate that the higher levels of LPMO products in reactions with impregnated feedstock are due to LPMO activity progressing for a longer time. Thus, higher product levels are being reached in reactions with impregnated feedstock before a gradual decline in Glc4gemGlc levels sets in at a later time point compared to the non-impregnated feedstocks (Figure 2). As reported earlier by Müller et al.,[49] upon cessation of Glc4gemGlc production, the apparent Glc4gemGlc yields slowly decrease over time due to the instability of this compound.

It is interesting to note the impact of the enzyme loading on Glc4gemGlc accumulation in the initial phase of the reactions (4−12 h). For feedstocks without 2-naphthol impregnation, the rate of Glc4gemGlc accumulation seemed to be less dependent of enzyme loading (except for the lowest loading), and the accumulation of LPMO products stopped earlier (Figure 2C,D). These observations suggest that in reactions with non-impregnated spruce, the LPMO reaction is limited by something else than the enzyme, for example, the availability of in situ generated H_2O_2. Such a scenario would entail that only a fraction of the LPMOs is needed to carry out co-substrate-limited cellulose degradation, and that gradual inactivation of the LPMOs only becomes noticeable when the level of catalytically competent LPMOs becomes limiting (i.e., earlier at lower enzyme loadings). Indeed, previous studies of LPMO activity during the degradation of cellulose with an LPMO-containing cellulase cocktail have led to proposing such scenarios.[49,50] On the other hand, for substrates with 2-naphthol impregnation, the enzyme loading had a more pronounced effect on Glc4gemGlc yields (compare Glc4gemGlc yields with various enzyme loadings at 4−24 h in Figure 2B,D), which suggests that in this case, the reaction

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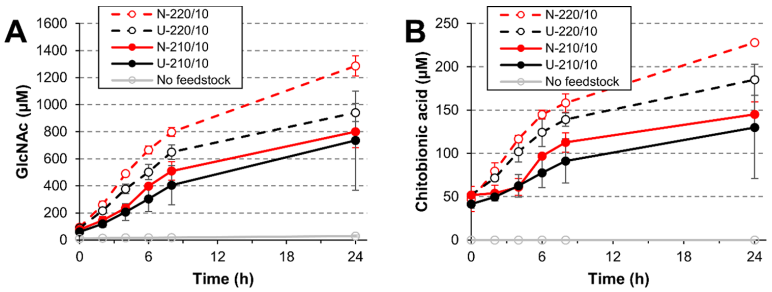

Figure 3. Comparison of the abilities of differently pretreated Norway spruce feedstocks to drive an LPMO reaction. Reactions containing 1% (w/v) β-chitin and 1 μM CBP21 in 50 mM Bis−Tris/HCl buffer (pH 6.5) were supplemented with 1% (w/v) of one of four differently pretreated spruce feedstocks, as indicated in the figure, and incubated at 40 °C. Pretreated feedstocks included steam-exploded spruce pretreated at 210 °C (U-210/10 and N-210/10) or 220 °C (U-220/10 and N-220/10) without (U-210/10 and U-220/10) or with (N-210/10 and N-220/10) 2-naphthol impregnation as indicated in Table S1. Soluble LPMO products (oxidized chito-oligosaccharides) were converted to *N*-acetylglucosamine (GlcNAc) and chitobionic acid with chitobiase (*Sm*GH20A) and quantified using HPLC. GlcNAc and chitobionic acid yields were calculated from at least three independent reactions, and standard deviations are shown as error bars.

was less limited by co-substrate availability and more by the amount of enzyme.

Importantly, the trends in apparent LPMO activity (Figure 2) correspond well to the trends in glucose solubilization (Figure 1). For the higher enzyme doses (4, 8, and 8 + 8 mg/g DM), the time when maximum Glc4gemGlc levels were recorded (Figure 2) coincides with the time when maximum glucose levels were recorded (Figure 1). This observation strongly suggests that the positive impact of 2-naphthol impregnation on cellulose saccharification is linked to LPMO performance.

Effect of 2-Naphthol Impregnation on the Capacity of Lignin to Drive the LPMO Reaction. It has been shown that lignin can reduce LPMOs and generate H_2O_2 (a co-substrate that leads to faster LPMO reactions).[33] It has also been shown that treatment of lignin with laccases, which changes the redox state of the lignin, affects the generation of H_2O_2 through reactions of lignin with O_2.[51] To study whether the positive impact of 2-naphthol impregnation on LPMO activity in Figure 2 is correlated with increased lignin reactivity and in situ H_2O_2 production, we assessed whether the various spruce feedstocks could promote the activity of a chitin-active LPMO (*Sm*AA10A, also known as CBP21) on β-chitin (the substrate of CBP21). CBP21 is inactive toward cellulose,[30,52] and the differences in the accumulation of oxidized chito-oligosaccharides are thus directly related to the ability of the lignin to donate electrons to the LPMO and generate H_2O_2. In this way, we were able to decouple effects on LPMO reactivity from other substrate factors like cellulose accessibility.

The accumulation of LPMO products in reactions of chitin-active CBP21 with β-chitin (Figure 3) followed a trend similar to that seen for the cellulose-active LPMOs present in Cellic CTec2 in reactions with pretreated spruce (Figure 2). Spruce samples pretreated at 220 °C were more potent in driving the CBP21 reaction on β-chitin than samples pretreated at 210 °C (compare dashed and solid lines in Figure 3). Furthermore, 2-naphthol impregnation of the spruce feedstock improved CBP21 activity, in particular for the reactions with feedstocks pretreated at 220 °C (compare red and black curves in Figure 3).

Previous studies have shown that diphenols and, even more so, methoxylated and methylated diphenols are good drivers of LPMO reactions, while monophenols, with their higher redox potentials, are not.[53,54] Interestingly, it has been shown that 2-naphthol impregnation of woody biomass (both hardwood and softwood) prevents free hydroxyl groups from taking part in cross-link formation during steam explosion,[10,19] which may lead to the preservation of reactive diphenolic structures during steam explosion and could explain the beneficial effect of 2-naphthol impregnation on the ability of lignin to drive LPMO reactions.

All in all, the results described above clearly show that the beneficial effect of 2-naphthol impregnation is not only due to the presumed improvement of cellulose accessibility and reduction of unspecific enzyme binding[11] but also to the enhanced reactivity of the lignin, which promotes LPMO activity. The CBP21 experiment shows that changes in the lignin structure during pretreatment (including the retention of phenolic OH groups; Figures S2 and S3) affect the H_2O_2-producing capacity of the feedstock and, consequently, the extent of LPMO activity.

Importantly, next to providing a novel explanation for the beneficial effect of 2-naphthol impregnation on biomass saccharification, the present results reveal that the impact of a (any) pretreatment technology on saccharification efficiency with modern LPMO-containing cellulase cocktails likely relates, at least in part, to the impact of the pretreatment on the redox state of the lignin. Thus, lignin-mediated effects on LPMO activity and on the overall performance of the LPMO-containing enzyme cocktail need to be considered when optimizing pretreatment steps and enzyme cocktails for biomass saccharification. Furthermore, the fact that optimal combinations of pretreatment and enzyme cocktails vary between feedstocks with different lignin content and composition may in part be explained by lignin-mediated effects on LPMO activity.

Impact of 2-Naphthol on the Saccharification Residue Assessed by SSNMR. To potentially gain a deeper understanding of the interaction of redox enzymes and the lignin fraction, we used SSNMR to analyze pretreated spruce samples (U-220/10 and N-220/10) before and after 48 h enzyme treatment with Cellic CTec2 at various enzyme doses (see the Supporting Information and Figure S7). While the extent of saccharification (Figure 1) was clearly reflected in a

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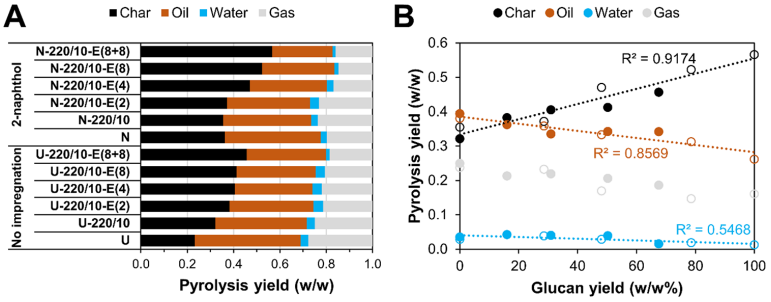

Figure 4. Product formation upon pyrolysis of selected spruce fractions. The graphs show yields of various pyrolysis products as a weight ratio of the total amount of products for native spruce without (U) or with (N) 2-naphthol impregnation, steam-exploded spruce without (U-220/10) or with (N-220/10) 2-naphthol impregnation, and saccharification residues of steam-exploded spruce treated with varying amounts (mg/g DM) of Cellic CTec2, E(2), E(4), E(8), and E(8 + 8). Gas yields were calculated as the mass difference of the feed and the sum of the char, water, and oil fractions. (B) shows the correlation between the extent of saccharification (i.e., glucan yield, as shown in Figure 1) and the proportion of pyrolysis products for U-220/10 (full symbols) and N-220/10 (open symbols); R^2 values were calculated for the combined data because of the similarity of the average compositions of U-220/10 and N-220/10.

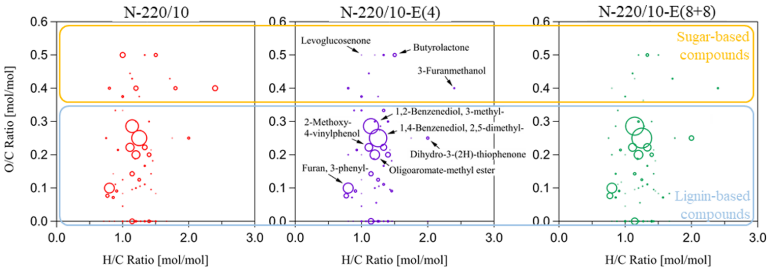

Figure 5. Van Krevelen plots for pyrolysis liquids obtained upon pyrolysis of selected spruce fractions with the dominant compounds assigned. The figure shows van Krevelen diagrams for pyrolysis liquids derived from a 2-naphthol-impregnated steam-exploded feedstock without (N-220/10) or with ca. 50% (N-220/10-E(4)) or ca. 100% (N-220/10-E(8 + 8)) cellulose conversion. Each circle corresponds to components with a specific chemical composition with respect to the O/C and H/C atomic ratio. The circle diameters correspond to the mass amounts of those components in the pyrolysis liquid. The components are classified into two groups of compounds, likely originating from cellulose and hemicellulose (top) and from lignin (bottom). Carbohydrates are characterized by an O/C atomic ratio of around 1, sugar-based derivatives commonly have an O/C ratio above 0.5, while lignins consist of phenolic structures with a significantly lower O/C ratio, commonly below 0.4.[58] The dominant products seem to be lignin-derived. Tracking the yields of individual compounds does not show clear trends due to the high complexity of the oil fraction.

decrease of the major cellulose peaks (at 73 ppm) relative to the lignin-specific peaks (at 130−155 and 55 ppm), potential changes in the lignin structure as a result of the enzyme treatments were apparently too low (relative to the total amount of lignin) to be detectable in the SSNMR spectra, even in the samples with close to 100% cellulose conversion (for details, see the Supporting Information).

Valorization of the Saccharification Residue Using Pyrolysis. A recent proof of concept study by Kalyani et al.[17] has shown that enzymatic saccharification and subsequent thermochemical conversion of the lignin-rich saccharification residue is a viable option for diversification of the product portfolio in a biorefinery. Here, we further explored this possibility by subjecting the feedstocks that had or had not been impregnated with 2-naphthol and their saccharification residues (48 h products from Figure 1C,D) to pyrolysis (Figure 4). Mass analysis of the resulting pyrolysis fractions showed that higher amounts of char and reduced amounts of pyrolysis liquids and noncondensables were obtained from the spruce feedstocks after steam explosion and, more so, after subsequent enzymatic saccharification with increasing enzyme dose (Figure 4A). This is in agreement with the observations by Kalyani et al.[17]

Comparison of the pyrolysis fractions derived from U and N shows that impregnation with 2-naphthol alone further contributes to char formation upon pyrolysis (Figure 4A). On the other hand, pyrolysis of the steam-exploded feedstocks (U-220/10 and N-220/10 in Figure 4A) yielded similar proportions of char (32.1 and 35.5% w/w, respectively), which is in line with the similar composition of these two feedstocks (for example, lignin contents of 40.6 ± 0.8 and 43.9 ± 0.6%, respectively; see Table S2). These data indicate that while 2-naphthol impregnation as such increased char formation (more char in N than in U), it limited the increase in char formation that occurs upon steam explosion (similar char in U-220/10 and N-220/10).

The overall picture emerging from Figure 4A is that processes that enrich the biomass in lignin led to an increase in char formation with a corresponding decrease in the amounts of pyrolysis liquid and noncondensable fractions. These observations are in agreement with previous studies indicating that the pyrolysis of pure lignin will lead to higher char and lower bio-oil yields than the pyrolysis of

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lignocellulosic biomass.[55,56] A closer look at the data for saccharification reactions with different enzyme loadings shows a clear trend between the extent of glucan solubilization (i.e., increased lignin content) and the size of the char (R^2 = 0.9174) and oil fractions (R^2 = 0.8569) for both the U-220/10 and N-220/10 series (Figure 4B).

Detailed analysis of the generated bio-oils showed a clear impact of 2-naphthol impregnation and the extent of cellulose conversion on the bio-oil composition. Complete lists of the identified compounds are provided in the Supporting Information. In general, 2-naphthol impregnation led to a higher proportion of furane-type and aromatic hydrocarbon-type compounds and a reduced proportion of acid/ester- and aldehyde/ketone-type compounds in the bio-oil when comparing the U- and N-series (for details, see the Supporting Information and Figure S8). Comparison of the van Krevelen diagrams of three samples, all 2-naphthol-impregnated samples, namely, N-220/10, N-220/10-E(4), and N-220/10-E(8 + 8), with 0, 48, and ca. 100% cellulose conversion, respectively, allowed us to identify effects on bio-oil composition that are related to the composition of the saccharification residue (Figure 5). Moving from left to right, i.e., toward reduced cellulose and hemicellulose content in the feedstock in Figure 5, the concentrations of sugar-derived furanics and anhydrosugar products in the oil were reduced correspondingly (see the top sections of the plots in Figure 5). Furthermore, with increasing lignin content (due to progressing cellulose conversion), the obtained pyrolysis oils became richer in monoaromatic phenols. While no selective increase in the fraction of individual phenolic compounds could be identified, the total concentration of the entire lignin-derived product spectrum increased with increasing cellulose removal (see the bottom sections of the plots in Figure 5).

All in all, these results show that the combination of 2-naphthol impregnation and steam explosion allows for a process that de facto splits highly recalcitrant spruce into a sugar fraction and a highly enriched lignin fraction that yields phenol-rich pyrolysis products, specifically biochar and bio-oils. Importantly, bio-oil quality is improved by the removal of polysaccharides prior to pyrolysis because this reduces the levels of unstable sugar-derived furanics, anhydrosugars, and acids that are prone to repolymerize and thus reduce storage stability. As a result, next to the efficient generation of glucose, a 2-naphthol-based process allows for the generation of bio-oil fractions with high aging stability and high phenolic content. Such fractions are suitable for the subsequent production of biobased resins and solvents.[57]

CONCLUSIONS

In this study, we demonstrated that impregnating spruce with 2-naphthol before steam explosion drastically enhanced the saccharification yields in reactions with the LPMO-containing cellulase cocktail Cellic CTec2 to the extent that close to 100% glycan conversion was reached. The positive impact of 2-naphthol impregnation was in part due to the beneficial effect of 2-naphthol impregnation on LPMO activity. This study also showed that changes in the lignin structure during pretreatment affected the H_2O_2-producing capacity of the feedstock and, consequently, the extent of LPMO activity and the overall performance of the LPMO-containing enzyme cocktail. It is important to recognize the hereby demonstrated large impact of the redox state of the lignin on LPMO activity and, consequently, the enzymatic saccharification efficiency.

As a consequence of the resulting high saccharification yields, 2-naphthol impregnation enables better separation of biomass components and may yield a relatively pure lignin fraction after enzymatic saccharification. A more efficient separation of biomass components has implications for downstream processing as exemplified by the valorization of the biomass solid residues after saccharification using pyrolysis. Here, we showed that the lignin and polysaccharide contents of the pretreated feedstocks and saccharification residues were in direct correlation with the ratios of the different pyrolysis fractions. Importantly, nearly complete removal of polysaccharides prior to pyrolysis clearly improved the quality of the bio-oil. Overall, our findings bring closer the prospect of an economically viable spruce-based biorefinery by combining biochemical and thermochemical conversion routes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.2c00286.

(Tables S1−S2) Complete list of pretreated feedstocks and their composition; (Figure S5) initial screening of these feedstocks for saccharification efficiency; (Figure S6) C4-oxidized cellobiose levels during enzymatic saccharification of the feedstocks pretreated at 210 and 220 °C; (Figures S1−S4 and S7) ^{13}C SSNMR ^{1}H-^{13}C cross-polarization spectra for the spruce fractions before and after pretreatment at 220 °C and enzymatic saccharification with various levels of enzyme dose; (Figure S8) composition of the pyrolysis oil fractions by compound type; detailed discussion of these additional experiments (PDF)

(Dataset S1) Detailed lists of compounds found in the pyrolysis oil fractions based on GC−MS data (XLSX)

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Author Contributions

The manuscript was written through the contributions of all authors as follows. L.D.H. was in charge of the conceptualization, investigation, formal analysis, visualization, original draft preparation, and review and editing of the manuscript. M.Ø. was in charge of the investigation and formal analysis. B.A. was in charge of the investigation, formal analysis, visualization, original draft preparation, and review and editing of the manuscript. R.T. was in charge of the conceptualization, funding acquisition, project administration, investigation, formal analysis, visualization, original draft preparation, and review and editing of the manuscript. V.G.H.E. was in charge of the conceptualization, supervision, funding acquisition, project administration, and review and editing of the manuscript. S.J.H. was in charge of the conceptualization, supervision, funding acquisition, project administration, and review and editing of the manuscript. A.V. was in charge of the conceptualization, supervision, project administration, formal analysis, visualization, original draft preparation, and review and editing of the manuscript. All authors have given approval to the final version of the manuscript.

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Notes

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■ ABBREVIATIONS

BG, β-glucosidase; DM, dry matter; GC, gas chromatography; GlcNAc, *N*-acetylglucosamine; FID, flame ionization detector; LPMO, lytic polysaccharide monooxygenase; MS, mass spectrometry; SSNMR, solid-state nuclear magnetic resonance

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Supplementary Information for

2-Naphthol impregnation prior to steam explosion promotes LPMO-assisted enzymatic saccharification of spruce and yields high-purity lignin

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Supplementary text

The impact of 2-naphthol impregnation and pretreatment temperature on feedstock composition

SSNMR analysis of untreated and 2-naphthol-impregnated native spruce showed an effect of 2-naphthol on lignin structure (**Fig. S1**). Impregnation of native spruce with 2-naphthol (without steam explosion) led to an apparent reduction in aromatic ring substituents, as shown by a decrease in the peaks corresponding to methoxy groups at 55 ppm, methyl groups linked to aromatic carbon at 20 ppm, and aromatic carbons carrying a substituent at around 150 ppm. Impregnation also led to a general decrease in signal intensity in the region 60 to 90 ppm. The latter indicates that lignin units became less condensed, given that the α-, β-, and γ-carbons in the aliphatic side chains of monolignols, which typically are involved in crosslinks, give peaks in this region.[44]

Changes in the SSNMR spectrum after steam explosion of the native spruce indicate depolymerization/cracking of lignin components (**Fig. S2**). A main feature is that the cellulose peaks, located in the 64-105 ppm range, are more resolved, probably due to transformation/loss of lignin compounds giving overlapping peaks. Around 80 ppm, there is a clear loss of intensity that can be assigned to loss of sp^2-C atoms in alkene units, and this loss may be related to the increased signal intensity in the aliphatic region (at 30-50 ppm). Furthermore, there is a slight but noticeable change in the aromatic region, at 110-150 ppm. The relative increase in signal intensity in the 115-135 ppm range indicates a relative increase in aromatic C-atoms without substituents. This is corroborated by the loss of methyl- and methoxy-groups at 20 and 55 ppm, respectively. The peak corresponding to carbonyl C-atoms (at 177 ppm) was also reduced by the steam explosion treatment.

Comparing SSNMR data for steam exploded spruce without (U-220/10) and with (N-220/10) 2-naphthol impregnation revealed several differences. The 2-naphthol-impregnated material showed a higher relative intensity of the peaks at around 150 ppm and 115-135 ppm, which reflect O-linked and non-substituted aromatic C-atoms, respectively (compare U-220/10 and N-220/10 in **Fig. S3**). Note that some of the aromatic C-atoms in the region around 120-130 ppm may originate from 2-naphthol as carbons in the naphthalene ring are expected to give a signal in that region.[11] Previously reported 2D-NMR analyses of pretreated aspen[10] and spruce[11] feedstocks showed that impregnation with 2-naphthol prior to steam explosion leads to a pretreated material where

naphthalene rings are incorporated in the lignin while phenolic hydroxyl groups are retained, both indicating a reduced degree of lignin condensation. Our data are in accordance with these findings.

Importantly, while steam explosion (**Fig. S2**) and the presence of 2-naphthol (**Figs. S1 & S3**) led to distinct changes in the lignin fraction, SSNMR showed that the cellulose structure was not affected and was of the cellulose I type in all samples (**Fig. S4**), as shown also earlier for spruce.[59,60]

Initial screening of the impact of 2-naphthol impregnation on enzymatic digestibility of spruce

To evaluate the impact of 2-naphthol impregnation on enzymatic digestibility, we subjected the feedstocks listed in **Table S1** to enzymatic saccharification with the LPMO-containing cellulase cocktail Cellic CTec2. Initial screening of all feedstocks showed that pretreatments with lower severity factors ($\log R_0$<4.0; all performed at temperatures up to 200 °C) led to low cellulose saccharification yields, independent of 2-naphthol impregnation. In these cases, the final glucose yields after 48 h were only 16-21% of the total glucan content. Improved saccharification yields and a positive effect of 2-naphthol impregnation could only be observed upon more severe pretreatment conditions (**Fig. S5**). Thus, we selected the four feedstocks pretreated at the highest severities (U-210/10, N-210/10, U-220/10, and N-220/10; see **Table S1**) for further experiments.

Changes in the biomass structure revealed by SSNMR

To gain a deeper understanding of the impact of the pretreatment and saccharification process on the resulting lignin, we used SSNMR to analyze pretreated spruce samples (U-220/10 and N-220/10) before and after 48 h enzyme treatment with Cellic CTec2 at various enzyme doses (**Fig. S7**). The extent of saccharification (see **Fig. 1** in the main manuscript) was clearly reflected in a decrease of the major cellulose peaks at 73 ppm relative to the lignin-specific peaks (130-155 and 55 ppm), and this effect is more visible for the reactions with 2-naphthol-impregnated feedstock, which gave higher degrees of saccharification (**Fig. S7**). Despite reaching nearly complete cellulose conversion, the samples treated with the highest dose of Cellic CTec2 still show remnants of cellulose.

Apart from the differences between U and N (**Fig. S1**) and U-220/10 and N-220/10 (**Fig. S3**) that are discussed above, **Fig. S7** indicates that the lignin structure, as judged from the relative intensities of the various lignin signals, was not affected by the enzymatic saccharification. While reactions between lignin and O_2 and reactions between lignin and the LPMO in Cellic CTec2 are to be expected, the level of these reactions is apparently too low (relative to the total amount of lignin) to be detectable in the SSNMR spectra (for details, see below).

It is well documented that LPMOs react with certain aromatic compounds carrying non-substituted phenolic hydroxyl groups, not only in a reduction reaction, where the lignin is oxidized to reduce the LPMO from its inactive Cu(II) to its active Cu(I) state, but also in a peroxidase-type reaction that consumes H_2O_2.[61] As an example, oxidation of the free hydroxylic group of the guaiacyl unit by an LPMO may yield a dienone structure, such as the guaiacyl-type spirodienone structure that has been observed in native spruce lignin.[62] This structure would give a distinctive ^{13}C NMR peak at 182.5 ppm,[62] which was not observed. One explanation for the lack of observed changes in the lignin structure could be that the actual number of changes is low, relative to the total amount of lignin. The LPMO reaction yielded around 1 mM C4-oxidized cellobiose after 48 h saccharification of N-220/10 with Cellic CTec2 at 8+8 mg/g DM loading, which corresponds to 0.36% of the released glucose units being oxidized (**Figs. 1,2**). Considering that the molecular weight of glucose (180 g/mol) and coniferyl alcohol, the most common lignin monomer in spruce, (180 g/mol) are similar, and if we only consider the net flow of electrons from lignin to the LPMO reaction, the corresponding level of oxidation in lignin would amount to 0.72% of lignin monomers being modified in N-220/10-E(8+8), and proportionally less in the solid residues of the other saccharification reactions (note that two electrons are needed per LPMO reaction). Considering this low percentage, it is not surprising that structural changes in the lignin could not be detected.

Valorization of the saccharification residue using pyrolysis

Pyrolysis of lignocellulose or a highly enriched lignin fraction (e.g., a lignin-rich saccharification residue), in general, yields phenol-rich pyrolysis products, specifically biochar and bio-oils. As a side-product, non-condensable (gaseous) products predominantly originate from sugar fractions. During pyrolysis, (lignin-derived) phenolic components tend to end up in the biochar due to secondary (recondensation) reactions. Accordingly, biochar formation did indeed increase as more cellulose had been removed (see **Fig. 4** in the main manuscript). On the other

hand, the oil fraction consists of both sugar- and lignin-derived compounds. To gain a better understanding of the impact of 2-naphthol impregnation and the extent of cellulose conversion on the bio-oil composition, we analyzed the generated bio-oils using GC-MS. The complete lists of the identified compounds are provided in **Supplementary Dataset 1**.

Fig. S8 shows the composition of the oil fractions based on the molecular structure and functional groups of the identified compounds. In general, 2-naphthol impregnation led to a higher proportion of furane-type and aromatic hydrocarbon-type compounds and a reduced proportion of acid/ester- and aldehyde/ketone-type compounds in the bio-oil, as can be seen by comparing the U- and N-series in **Fig. S8**. Notably, there were no clear correlations between cellulose conversion yields and the proportion of the various pyrolysis components (**Fig. S8**), indicating that, although the bio-oil yield depended on the carbohydrate content (see **Fig. 4B** in the main manuscript), the composition of the oil phase of the pyrolysis liquids was less affected. One explanation for this is that most carbohydrate-derived compounds require derivatization prior to GC analysis in order to increase their volatility. This regards specifically for monomeric sugars, anhydrosugars and sugar acids.[63] Hence, they are not detected in the current GC-MS analysis used to determine the composition of the oil. The GC-MS analysis only allows detection and quantification of volatile components, such as furfural or hydroxymethyl furfural, which are examples of carbohydrate-derived volatile compounds. Moreover, a large portion of carbohydrate-based compounds are predominantly present in the aqueous, and not the organic, phase. On a general level, lower amounts of detectable carbohydrate-derived products, such as furanes, were identified in the bio-liquids, as shown in **Fig. S8**. This can be explained by the reduced carbohydrate content of the saccharification residues due to increased cellulose conversion. It is noteworthy that by increasing the enzyme dose and impregnating the biomass with 2-naphthol prior to steam explosion, we were able to produce a quasi-pure lignin fraction from spruce wood (see **Fig. 1D**, N-220/10-E(8+8), in the main manuscript).

The results presented in **Fig. 5** of the main manuscript and **Fig. S8** corroborate earlier findings that pyrolysis of pure lignin yields primarily phenolic components. Pyrolysis of lignocellulosic biomass and saccharification residues containing higher amounts of residual polysaccharides yields a range of sugar derivatives, in particular, furanics, acids and anhydrosugars, along with lignin-based phenolics.[55]

Figure S1

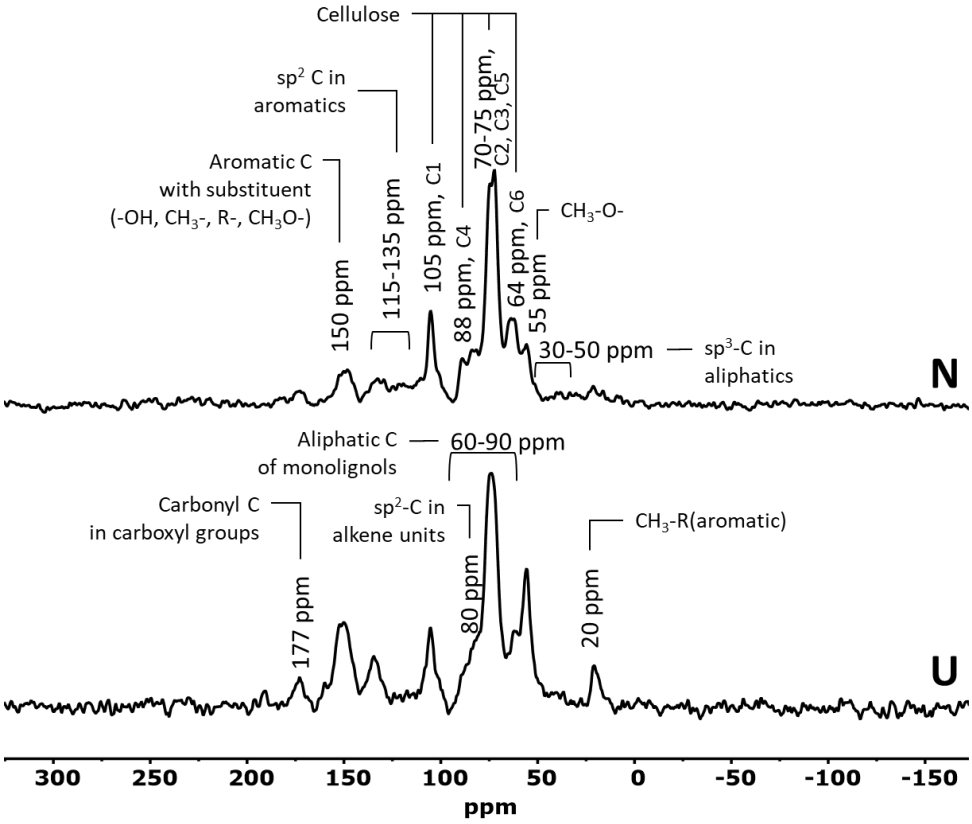

Figure S1. ^{13}C SSNMR ^{1}H-^{13}C cross-polarization spectra of native spruce without (U) or with (N) 2-naphthol impregnation. The spectra were normalized based on the highest cellulose peak (at 73 ppm; the second major cellulose peak appears at 105 ppm).

Figure S2

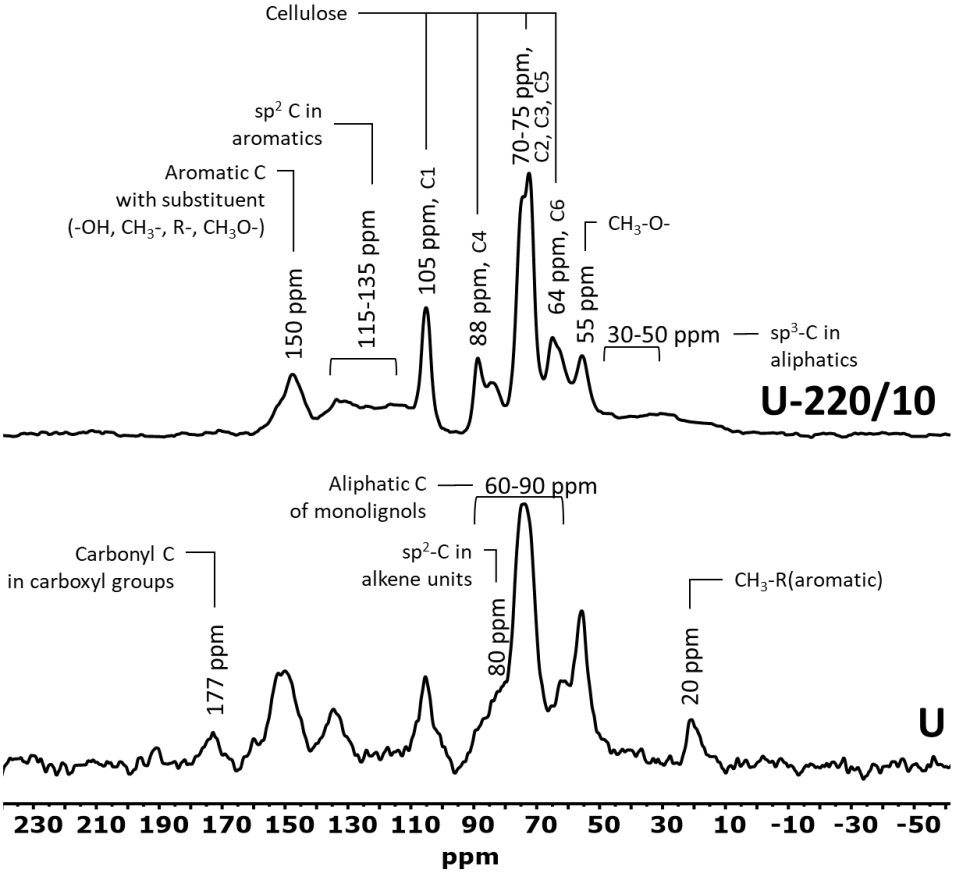

Figure S2. ^{13}C SSNMR ^{1}H-^{13}C cross-polarization spectra of native spruce (U) and steam-exploded spruce (U-220/10), both without 2-naphthol impregnation. The spectra are normalized on the highest cellulose peak, at 73 ppm.

Figure S3

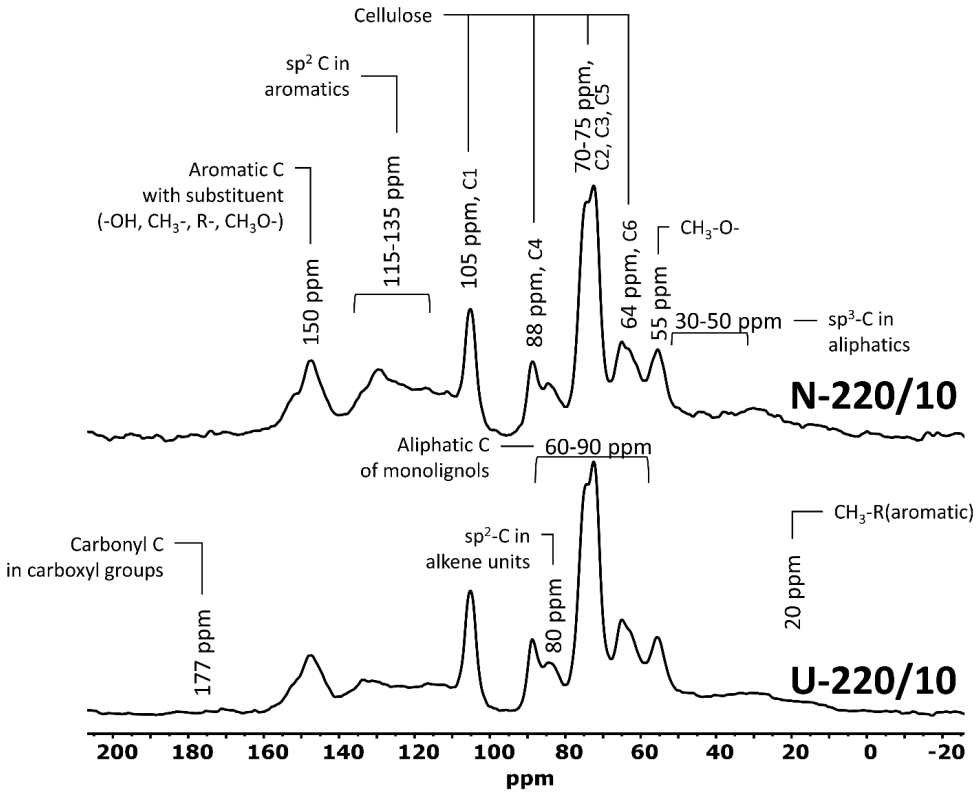

Figure S3. ^{13}C SSNMR ^{1}H-^{13}C cross-polarization spectra of steam-exploded spruce without (U-220/10) or with (N-220/10) 2-naphthol impregnation. The spectra were normalized based on the highest cellulose peak (at 73 ppm). The two spectra differ mainly in the signals corresponding to O-linked aromatic C-atoms at about 150 ppm and to non-substituted aromatic C-atoms at 115-135 ppm, both of which have higher intensity in N-220/10 than in U-220/10.

Figure S4

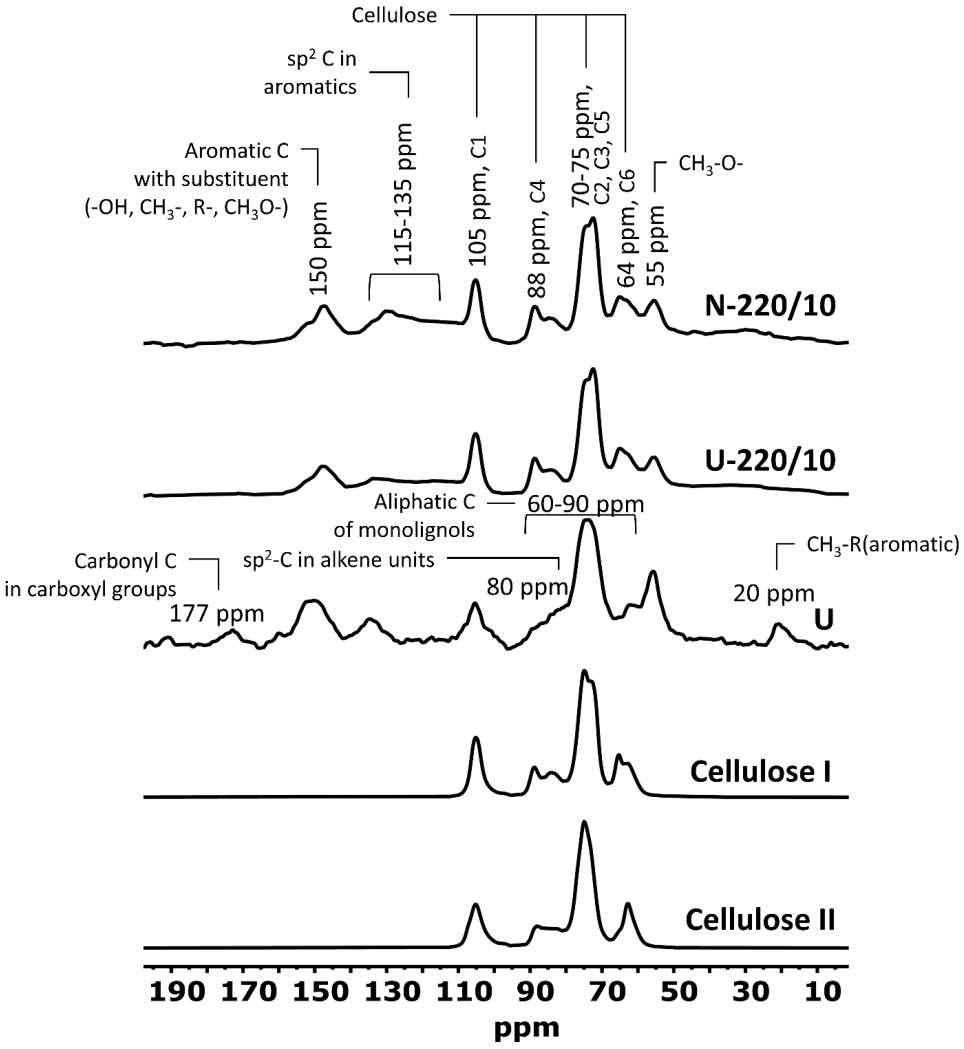

Figure S4. ^{13}C SSNMR ^{1}H-^{13}C cross-polarization spectra of native spruce (U) and steam-exploded spruce without (U-220/10) or with (N-220/10) 2-naphthol impregnation. The spectra were normalized based on the highest cellulose peak (at 73 ppm) and are shown together with spectra for Cellulose I_β (the native form of cellulose) and Cellulose II as reference. Peaks corresponding to the carbons of glucose units in cellulose and peaks corresponding to C atoms in lignin are labeled. Note that the upper two spectra are identical to those shown in **Fig. S3**.

Figure S5

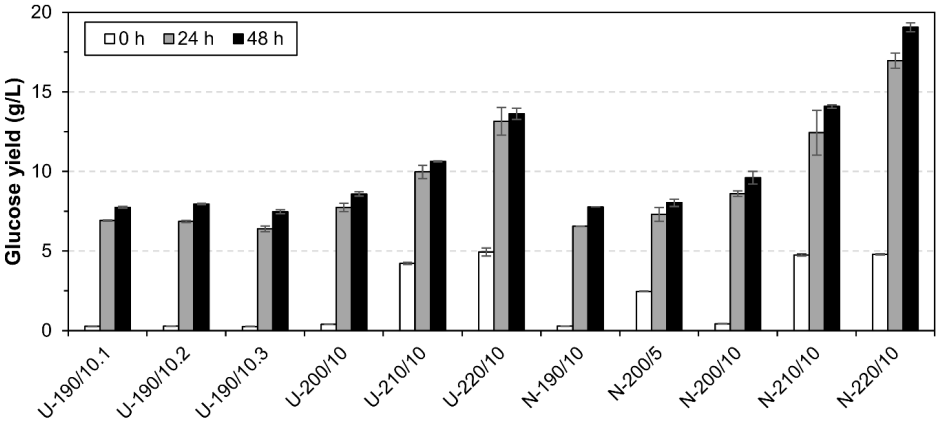

Figure S5. Initial screening of saccharification efficiency for differently pretreated spruce feedstocks. Reaction mixtures (with 20 mL total volume) contained 10% DM (w/v) pretreated feedstock and 4 mg/g DM Cellic CTec2 in 50 mM sodium acetate buffer (pH 5.0) at 50 °C. Reactions were carried out in 50 mL glass bottles sealed with rubber septa, and 300 µL samples were withdrawn with a 1 mL syringe after opening the caps at each sampling point. Glucose yields were calculated from at least two independent reactions and standard deviations are shown as error bars; for N-190/10 only one reaction was conducted. Note that steam explosion at 190 °C for 10 min (generating U-190/10.1-3 samples) was run three times, underpinning reproducibility. The maximum theoretical yield of glucose in these experiments was 42-54 g/L. The relatively low yields are in part due to acidification of the samples, which happened because the starting pH was not adjusted, in contrast to the experiments reported in the main manuscript. For Sample IDs and pretreatment conditions, see **Table S1**.

Figure S6

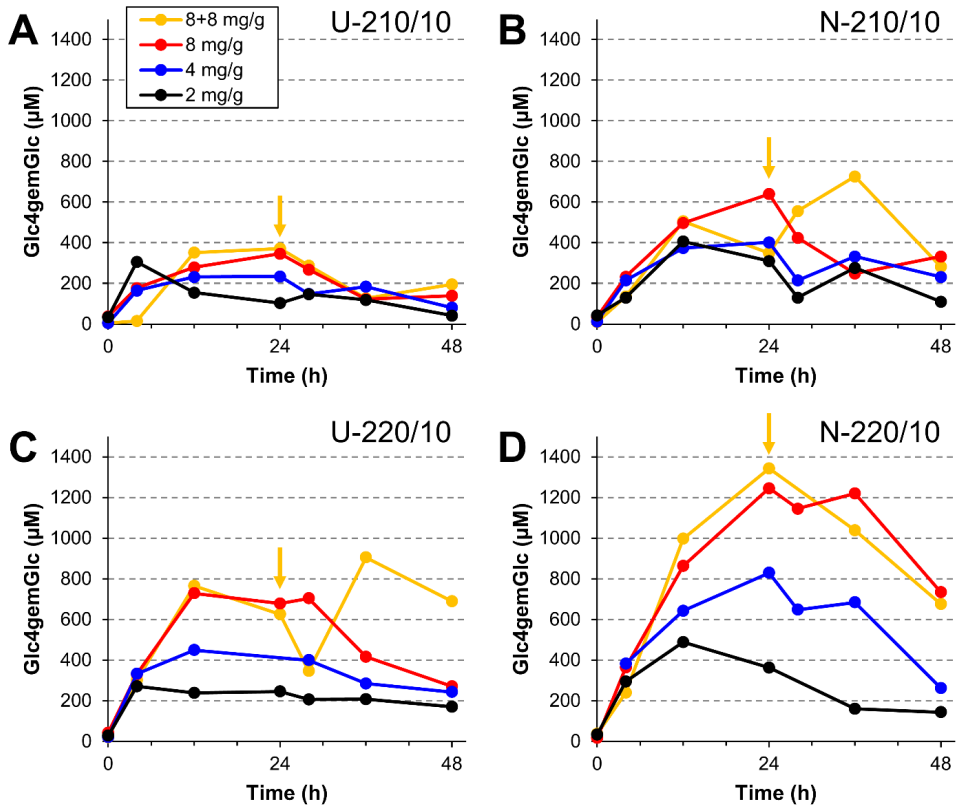

Figure S6. Glc4gemGlc (C4-oxidized cellobiose) levels during enzymatic saccharification of pretreated Norway spruce. Norway spruce pretreated at 210 °C **(A, B)** or 220 °C **(C, D)** without impregnation **(A, C)** and with 2-naphthol impregnation **(B, D)** was subjected to saccharification with Cellic CTec2 at various enzyme loadings. In the reactions, 10% DM (w/v) substrate was incubated with 2 (black), 4 (blue) or 8 (red) mg/g DM Cellic CTec2 in 50 mM sodium acetate buffer pH 5.0 at 50 °C. In the reaction marked as "8+8 mg/g" (orange), 8 mg/g DM Cellic CTec2 was added at *t*=0 h, and an additional 8 mg/g DM Cellic CTec2 was added at *t*=24 h (marked with orange arrow). The graphs show the Glc4gemGlc values measured for one of the three parallel reactions shown in **Fig. 1** of the main manuscript; similar data for one of the other reactions appear in **Fig. 2** of the main manuscript. These results are shown separately because the various samples were analyzed on different HPAEC-PAD systems that show different performances when it comes to the quite complicated and hard to precisely reproduce analysis of C4-oxidized products. The

graphs shown here and in **Fig. 2** show similar trends, both when comparing absolute Glc4gemGlc levels as well as regarding the differences between the substrates and enzyme dosages.

Figure S7

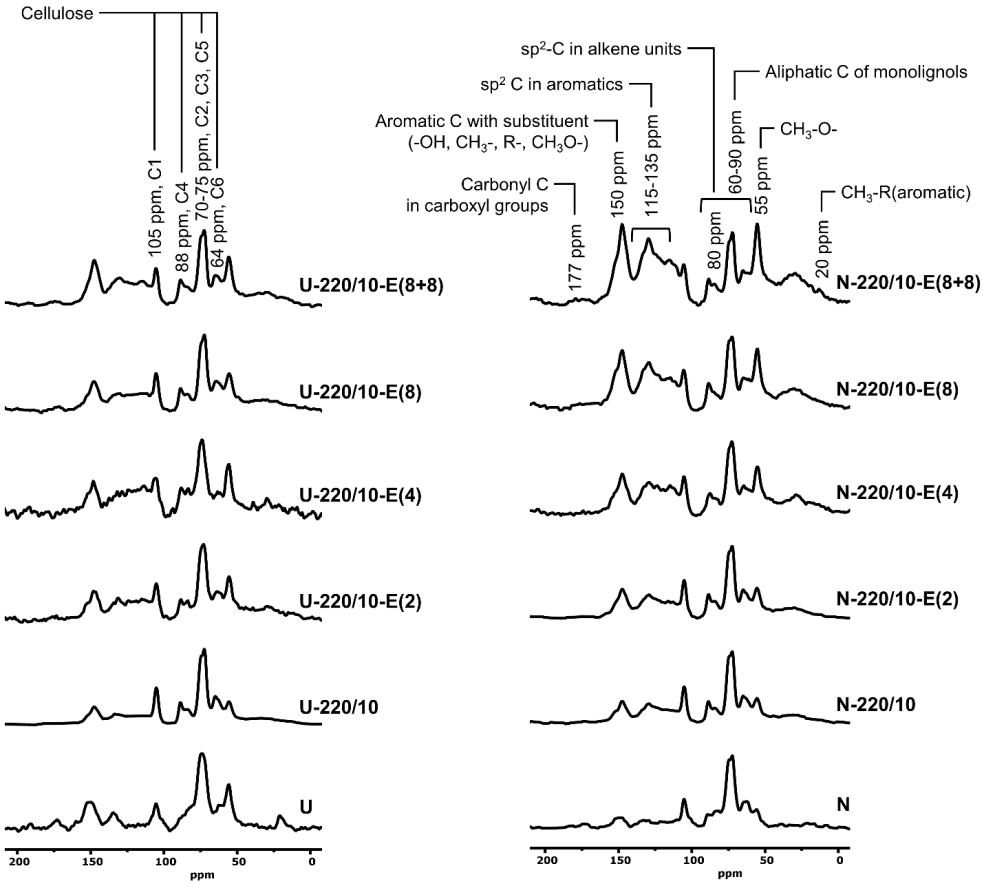

Figure S7. ^{13}C SSNMR ^{1}H-^{13}C cross-polarization spectra of native and pretreated spruce and saccharification residues. Samples include native spruce without (U) or with (N) 2-naphthol impregnation, steam-exploded spruce without (U-220/10) or with (N-220/10) 2-naphthol impregnation, and saccharification residues of steam exploded spruce treated with varying amounts (mg/g DM) of Cellic CTec2 (E(2), E(4), E(8), and E(8+8)). The spectra in each column are normalized based on the highest cellulose peak at 73 ppm. Cellulose-specific peaks are marked above the left panel; lignin-specific peaks are marked above the right panel.

Figure S8

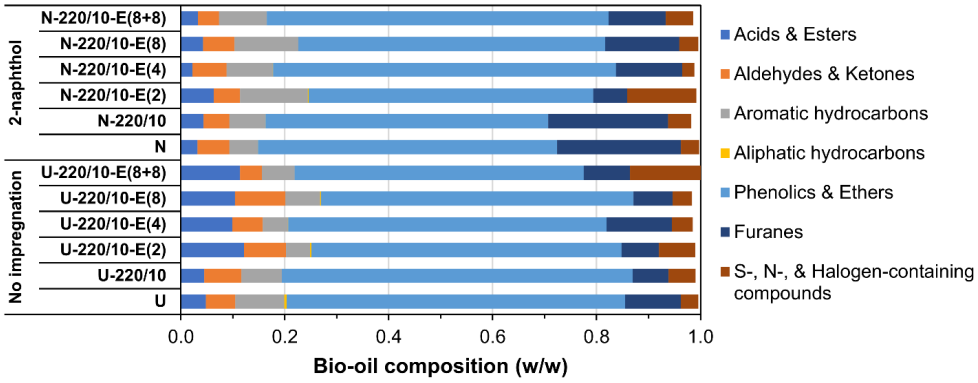

Figure S8. The composition of the generated pyrolysis oils by compound type. The graph shows the composition of the bio-oils as a weight ratio of the total mass of the oil fraction prepared from native spruce without (U) or with (N) 2-naphthol impregnation, steam-exploded spruce without (U-220/10 or with (N-220/10) 2-naphthol impregnation, and saccharification residues of steam exploded spruce treated with varying amounts (mg/g DM) of Cellic CTec2 (E(2), E(4), E(8), and E(8+8)). The underlying GC-MS data are provided in **Supplementary Dataset 1**.

Supplementary tables

Table S1. Steam explosion conditions used. The 2-naphthol concentration for impregnated samples was 0.2 M.

Sample ID	Impregnation	Temperature (°C)	Residence time (min)	Severity factor logR_0
U-190/10.1	–	190	10	3.65
U-190/10.2	–	190	10	3.65
U-190/10.3	–	190	10	3.65
U-200/10	–	200	10	3.94
U-210/10	–	210	10	4.24
U-220/10	–	220	10	4.53
N-190/10	2-naphthol	190	10	3.65
N-200/5	2-naphthol	200	5	3.64
N-200/10	2-naphthol	200	10	3.94
N-210/10	2-naphthol	210	10	4.24
N-220/10	2-naphthol	220	10	4.53

Table S2. Compositional data for the pretreated feedstock samples. Sample IDs are explained in **Table S1**.

Sample ID	Glucan (% of DM)	Xylan (% of DM)	Mannan (% of DM)	Galactan (% of DM)	Arabinan (% of DM)	Lignin (% of DM)
U-190/10.1	44.2±1.4	4.3±0.3	11.6±0.8	0.6±0.0	0.0±0.0	30.0±0.5
U-190/10.2	45.2±32.1	4.6±0.6	11.9±1.1	0.9±0.3	0.0±0.0	28.7±0.6
U-190/10.3	41.3±0.8	4.0±0.0	10.8±0.5	0.6±0.0	0.0±0.0	30.8±0.9
U-200/10	39.0±2.1	3.2±0.6	8.9±1.3	0.6±0.0	0.0±0.0	32.0±0.0
U-210/10	37.9±2.5	2.3±0.0	6.3±0.3	0.5±0.0	0.0±0.0	39.1±2.1
U-220/10	40.8±2.2	0.7±0.0	3.4±0.0	0.0±0.0	0.0±0.0	40.6±0.8
N-190/10	38.6±1.9	4.7±0.3	9.3±0.3	1.2±0.3	0.7±0.3	29.9±0.2
N-200/5	38.6±1.4	4.8±0.3	10.8±1.1	1.3±0.3	0.5±0.0	31.6±0.5
N-200/10	40.9±5.2	3.9±0.6	10.7±1.4	1.0±0.0	0.2±0.3	32.6±0.8
N-210/10	48.8±0.0	2.7±0.0	7.3±0.4	0.6±0.0	0.0±0.0	36.9±0.7
N-220/10	41.8±5.8	0.2±0.4	2.1±0.4	0.0±0.0	0.0±0.0	43.9±0.6

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Paper 3

H_2O_2 feeding enables LPMO-assisted cellulose saccharification during simultaneous fermentative production of lactic acid

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H_2O_2 feeding enables LPMO-assisted cellulose saccharification during simultaneous fermentative production of lactic acid

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Abstract

Biocatalytic production of biochemicals from lignocellulosic materials can proceed via two main routes, referred to as separate hydrolysis and fermentation (SHF), a two-step process, and simultaneous saccharification and fermentation (SSF), a one-pot process. Because the fermentation process typically is anaerobic, oxidative enzymes found in modern commercial cellulase cocktails, such as cellulose-active lytic polysaccharide monooxygenases (LPMOs), may be inhibited, limiting the overall efficiency of the enzymatic saccharification. Therefore, it has been suggested that SSF approaches are incompatible with harnessing the power of the LPMOs. Recent discoveries, however, have shown that LPMOs do not per se require dioxygen, and that LPMOs are active under anoxic conditions if they are provided with hydrogen peroxide at low concentrations. In this study, we build on this concept and investigate the potential of using externally added H_2O_2 to sustain oxidative cellulose depolymerization by LPMOs during an SSF process for lactic acid production. The results of bioreactor experiments with 100 g/L cellulose clearly show that continuous addition of small amounts of H_2O_2 (at a rate of 90 μM/h) during SSF enables LPMO activity and improves lactic acid production. While further process

optimization is needed, the present proof-of-concept results show that modern LPMO-containing cellulase cocktails such as Cellic CTec2 can be used in SSF setups, without sacrificing the LPMO activity in these cocktails.

Keywords: biorefinery; simultaneous saccharification and fermentation; lytic polysaccharide monooxygenase; LPMO; hydrogen peroxide; lactic acid

Introduction

Processing of lignocellulosic biomass to a spectrum of materials, fuels, and chemicals has been well explored during the past decades. Lignocellulose-based products include ethanol and value-added chemicals such as lactic acid, obtained via saccharification and fermentation, and cellulosic products such as nanocellulose (Chandel et al., 2018; Rosales-Calderon & Arantes, 2019). Biochemical conversion processes based on saccharification consist of three main steps: 1) pretreatment of the feedstock to increase cellulose accessibility, 2) enzymatic depolymerization of the plant polysaccharides to monomeric sugars, and 3) microbial fermentation of the sugars into value-added biochemicals. The last two steps can, in general, be performed either sequentially, referred to as separate hydrolysis and fermentation (SHF), or simultaneously, in a simultaneous saccharification and fermentation (SSF) setup. The potential advantages of SSF over SHF include reduced product inhibition of cellulases due to the lack of glucose accumulation, higher process productivity, and reduced process costs of a one-pot operation (Ishizaki & Hasumi, 2014; Wingren et al., 2003). The disparity between the temperature and pH optima of the enzymes and fermenting strains, on the other hand, can be a

disadvantage of SSF. For example, SSF often requires low operating temperatures, which may lead to reduced efficiency of the enzyme cocktail and increased risk of microbial contamination. Using thermophilic strains that are capable of growing at the optimal temperature of cellulase cocktails (about 50 °C), such as the lactic acid-producing *Bacillus coagulans*, may reduce contamination risks and enable efficient saccharification (John et al., 2009).

Commercial cellulase cocktails include cellulases (cellobiohydrolases, endoglucanases, and β-glucosidases), hemicellulases, and recently also lytic polysaccharide monooxygenases (LPMOs) (Harris et al., 2014; Johansen, 2016; Merino & Cherry, 2007). In the past decades, the depolymerization of lignocellulosic polysaccharides by hydrolases (including hemicellulases) has been optimized extensively (Harris et al., 2014; Østby et al., 2020). The discovery of oxidative polysaccharide degradation in 2010 (Vaaje-Kolstad et al., 2010) and its importance for not only chitin (Vaaje-Kolstad et al., 2010) but also cellulose depolymerization (Chylenski et al., 2019; Hemsworth et al., 2015; Johansen, 2016) has shifted focus towards this aspect of enzymatic biomass degradation. LPMOs contain a single active-site copper that, when reduced, allows for oxidative cleavage of β-1,4-glycosidic bonds in crystalline cellulose using either a monooxygenase reaction (involving O_2 and stoichiometric amounts of reductant) or a peroxygenase reaction (involving H_2O_2 and "priming" amounts of reductant) (Bissaro et al., 2018; Chylenski et al., 2019; Hedegård & Ryde, 2018; Hedison et al., 2021). Of note, it has been suggested that the monooxygenase reaction may not occur, and that LPMO activity observed under "monooxygenase conditions" reflects a peroxygenase reaction that is limited by H_2O_2 that is generated *in situ* through oxidation of the reductant.

The need for an oxygen-containing co-substrate (O_2 or H_2O_2), to allow for LPMO activity, has considerable implications for biomass processing with LPMO-containing cellulase cocktails.

The apparent O_2 dependency of today's state-of-the-art, LPMO-containing cellulase cocktails has urged the field to reconsider process setups focusing on the delivery of oxygen to the reaction slurry (Johansen, 2016; Müller et al., 2017). Although aeration during the saccharification step facilitates LPMO reactions in an SHF process, LPMO reactions are limited in SSF processes, due to competition for (di)oxygen between the LPMOs in the cellulase cocktail and the fermenting microorganism (Cannella & Jørgensen, 2014; Müller et al., 2017) or because the fermentation is anaerobic. Anaerobic fermenting microorganisms will be hampered by the oxidative stress caused by O_2 and reactive oxygen species that may emerge during oxidative polysaccharide degradation (Ayer et al., 2014). SSF experiments with modern LPMO-containing cellulase cocktails in which low amounts of O_2 were added to drive the LPMO reaction showed 20%–30% lower overall yields compared to the analogous SHF process for ethanol- (Cannella & Jørgensen, 2014) and lactic acid-fermenting (Müller et al., 2017) strains, underpinning the challenges related to combining LPMO activity and anaerobic fermentation.

The recent discovery that LPMOs are efficient peroxygenases (Bissaro et al., 2017) opens up new possibilities for controlling LPMO activity in SSF processes. H_2O_2 may be generated *in situ* through abiotic reactions between molecular O_2 and reducing compounds, including lignin (Kont et al., 2019; Rieder et al., 2021; A. A. Stepnov et al., 2022; Stepnov et al., 2021), or through the (off-pathway) oxidase activity of LPMOs (Kittl et al., 2012; Stepnov et al., 2021), or be supplied externally in a controlled manner, e.g. by feeding a (diluted) H_2O_2 solution into a reactor (Müller et al., 2018). The possibility to add H_2O_2 in a liquid form rather than to rely on aeration could solve the problems that come with oxygen delivery to the system, including low oxygen transfer rates and limited solubility and diffusion of oxygen in a high-consistency slurry. Furthermore, feeding H_2O_2, rather than relying on *in situ* generation of H_2O_2 through reactions with O_2, opens

the perspective of fueling the LPMO reaction under anaerobic conditions. A positive impact of driving the LPMO reaction with externally added H_2O_2 on cellulose saccharification has been proven both at laboratory (Bissaro et al., 2017; Müller et al., 2018) and demonstration scale (Costa et al., 2020). Importantly, in the presence of substrate, LPMOs have low micromolar affinities for added H_2O_2 (Kuusk et al., 2018; Rieder et al., 2021), which means that externally added H_2O_2 is quickly consumed by LPMOs, and that steady-state H_2O_2 concentrations are in the low micromolar range (Kadić et al., 2021). The high affinity for H_2O_2 also means that LPMOs likely can compete with the H_2O_2-handling systems of microbes (Seaver & Imlay, 2001), such as microbial catalases, which are fast enzymes but tend to have low affinities for H_2O_2 (Bissaro et al., 2017). Considering the above, it seems feasible to develop anaerobic SSF processes with H_2O_2 feeding that keeps the H_2O_2 concentration at a micromolar level that is high enough to drive the LPMO reaction, while not harming microbial fermentation.

Here, we present proof-of-concept studies aimed at investigating whether H_2O_2 can drive LPMO reactions in an SSF process, with cellulose (Avicel) as substrate, using the LPMO-containing cellulase cocktail Cellic CTec2 for saccharification and thermophilic lactic acid-producing *Bacillus coagulans* for fermentation. The similar temperature optima of the cellulase cocktail and *B. coagulans* (50 °C) enabled us to directly compare lactic acid yields and LPMO products in the various process setups since all steps were run at 50 °C. Thus, we assessed if SSF setups with H_2O_2 feeding could be a feasible alternative for one-pot conversion of cellulose to lactic acid.

Materials and methods

Substrate and enzymes

The substrate used was microcrystalline cellulose (Avicel PH-101) from Sigma (St. Louis, MO, USA). Cellic CTec2 (CC2), Celluclast 1.5 L and β-glucosidase were provided by Novozymes A/S (Bagsværd, Denmark). Protein concentrations of the enzyme preparations were determined using the Bradford method with bovine serum albumin as a standard (Bradford, 1976). In reactions with a Celluclast and β-glucosidase (CelBG) mixture, Celluclast and β-glucosidase were mixed in a 9:1 (w/w) ratio based on amounts of protein.

Strain and growth media

Bacillus coagulans (also called *Weizmannia coagulans*) strain DSM 2314 was purchased from DSMZ German Collection of Microorganism and Cell Cultures (Braunschweig, Germany). The strain was grown on Tryptic Soy Broth (TSB) medium, containing 20 g/L D-Glucose (Sigma Aldrich, St.Louis, MO, USA), 10 g/L Bacto™ Tryptone (Gibco, Detroit, MI, USA), 5 g/L Difco™ Soytone (Gibco, Detroit, MI, USA), 3 g/L NaCl (Sigma Aldrich, St. Louis, MO, USA), 2 g/L K_2HPO_4 (Sigma Aldrich, St. Louis, MO, USA), and 1 g/L Tween 80 (Sigma Aldrich, St. Louis, MO, USA) at pH 6.5, with 1% Agar (VWR, Radnor, PA, USA) for plates.

Saccharification setup

Enzymatic saccharification of Avicel was carried out in 1.5 L Minifors 2 bioreactors (Infors HT, Bottmingen, Switzerland) with a final working volume of 1 L. The reactions contained 10% (w/v) Avicel, supplemented with 10 g/L Bacto™ Tryptone, 5 g/L Difco™ Soytone, 3 g/L NaCl

and 1 g/L Tween 80 antifoam. In the reactors, 50 mM sodium acetate buffer (pH 5.5) was used as buffer instead of K_2HPO_4. Saccharification reactions were carried out at 50 °C with stirring at 300 rpm using two sets of Rushton impellers installed on a high torque motor. For anaerobic reactions, the reactors were sparged with N_2 at a flow rate of 0.2 L/min for 2 h before addition of enzymes, and the N_2 flow was maintained at 0.1 L/min throughout the reaction. For aerobic reactions, oxygen was delivered to the reaction by diffusion from air-containing headspace. L-Ascorbic acid (Sigma Aldrich, St. Louis, MO, USA) (AscA; 1 mM final concentration) was added at t=0 h, immediately before adding 50 mL enzyme solution (Cellic CTec2 or CelBG), with a final enzyme dosage of 4 mg/g substrate, to start the reaction. For reactions with H_2O_2 feeding, a 30% (w/w) H_2O_2 solution (Sigma Aldrich, St. Louis, MO, USA) was diluted in sterile Milli-Q water. For feed rates of 40, 80, and 160 µM/h, solutions with 67.5, 135, and 270 mM H_2O_2, respectively, were prepared; and these solutions were supplied to the reactor using a syringe pump (Atlas syringe pump with 50 µL syringe, Syrris, Royston, United Kingdom) at 600 µL/h. Samples (1 mL each) were withdrawn periodically using a Super Safe Sampler (Infors HT, Switzerland) and boiled for 15 min to inactivate the enzymes. All samples were stored at -20 °C before analysis.

Inoculum preparation

Inoculum was prepared in two steps. First, *B. coagulans* was grown for ca. 24 h on a TSB agar plate at 50 °C, before being transferred into 50 mL TSB liquid medium in a 250 mL Erlenmeyer flask. After incubation at 50 °C with orbital shaking at 160 rpm for ca. 12 h, this preculture was used to inoculate four 2 L Erlenmeyer flasks containing 300 mL TSB medium each with a starting OD_{600} of 0.1. The main cultures were incubated at 50 °C with orbital shaking at 160 rpm

(Multitron Standard, Infors HT, Bottmingen, Switzerland) for 4 h before harvesting the cells. Harvesting was done by spinning down the cultures separately at 4,149 *g* for 15 min at 4 °C, after which the pellets were washed with 50 mL sterile NaCl solution (0.9%, w/v) and spun down again at 4,149 g for 15 min at 4 °C. The washed pellets were each resuspended in 30 mL sterile NaCl solution (0.9%, w/v), pooled and used to inoculate the fermenters. OD_{600} of the cell suspension was measured and used to calculate inoculation volume required to reach a starting OD_{600} of 0.5. Total inoculation volume was 50 mL with sterile Milli-Q water making up any remaining volume.

SHF and SSF setups

A two-step process referred to below as separate hydrolysis and fermentation (SHF), entailing a saccharification step followed by a combined saccharification and fermentation step, was started in 1.5 L Minifors 2 bioreactors (Infors HT, Switzerland) with a saccharification step as outlined above using the same substrate and media. After 24 h, inoculum (a cell suspension in 0.9 % NaCl, with a total volume 50 mL) was added aseptically to obtain a starting OD_{600} of 0.5. Sampling and sample handling were done as described above for the saccharification setup.

Simultaneous saccharification and fermentation (SSF) was carried out in 1.5 L Minifors 2 bioreactors (Infors HT, Switzerland) essentially as described in the Saccharification experiments, with the difference that the reactions were started by adding aseptically 50 mL enzyme solution (4 mg/g substrate) and 50 mL inoculum (starting OD_{600} was 0.5) immediately thereafter. Sampling and sample handling were done as described above for the Saccharification setup.

Table 1 provides an overview of the various process setups used in this study.

Analysis of saccharification and fermentation products

Saccharification and fermentation samples were analyzed for glucose, cellobiose and lactic acid by high performance liquid chromatography (HPLC) using a Dionex Ultimate 3000 (Dionex, Sunnyvale, CA, USA) with a Rezex ROA-organic H+ (8%) 300×7.8 mm analytical column (Phenomenex, Torrance, CA, USA) operated at 65°C and connected to a Shodex RI-101 differential refractive index detector (Shodex, Japan) (Müller et al., 2015). The eluent was 5 mM H_2SO_4, with a flow rate of 0.6 mL/min. Glucose, cellobiose, and lactic acid standards were obtained from Sigma Aldrich (St. Louis, MO, USA). All samples were diluted and filtered (96-well filter plate with 0.45 µm pore size; Millipore, Burlington, MA, USA) prior to HPLC/HPAEC analysis.

Saccharification and fermentation samples were analyzed for the dominating LPMO product, C4-oxidized cellobiose (Glc4gemGlc) by high performance anion-exchange chromatography (HPAEC) using a Dionex ICS-5000 system (Dionex, Sunnyvale, CA, USA) with a CarboPac PA200 analytical column (3×250 mm) and guard column (3×50 mm) at 30 °C, connected to a pulsed amperometric detector (PAD), using a 39 min multistep gradient as reported earlier (Hegnar et al., 2021). Glc4gemGlc standards were produced from cellopentaose (Megazyme, Wicklow, Ireland) as described by Müller et al. (Müller et al., 2015). Note that the LPMOs in Cellic CTec2 are predominantly C4-oxidizing and that the cellulases in the enzyme cocktail convert longer LPMO products to the oxidized dimer, which explains why only Glc4gemGlc was analyzed.

Results and discussion

Cellulose saccharification with H_2O_2 supply

To establish a baseline for glucose release from cellulose during SHF and SSF processes, saccharification reactions were run at 1 L scale with varying H_2O_2 feed rates (40–160 µM/h) under anoxic conditions (N_2 atmosphere) or without H_2O_2 feeding in the presence of air (**Figure 1**). In all reactions except when aerated (CC2_Air), an anoxic atmosphere was maintained to enable comparability with the anoxic fermentations in the SHF and SSF process setups. The results of these reactions, presented in **Figure 1**, corroborate earlier observations that continuous supply of H_2O_2 boosts saccharification of cellulose with LPMO-containing cellulase cocktails (Costa et al., 2020; Kadić et al., 2021; Müller et al., 2018). Maximum glucose concentration at 48 h (72.5 g/L) was achieved in the reactions with Cellic CTec2 and the 80 µM/h H_2O_2 feed rate (**Figure 1A**). Increasing the H_2O_2 feed rate beyond 80 µM/h, i.e. to 160 µM/h, led to higher LPMO activity (**Figure 1B**) but a decline in the final glucose yield (**Figure 1A**). This decline in glucose yield indicates enzyme inactivation, presumably due to too high H_2O_2 levels and/or depletion of ascorbic acid, as shown earlier by Kadić et al. (Kadić et al., 2021). Enzyme inactivation is also clear for the LPMO product levels: the LPMO product is unstable and levels will decline over time if the LPMOs are inactivated while still adding H_2O_2, as is indeed observed for the reaction with the 160 µM/h H_2O_2 feed rate, starting at 30 h. It has recently been suggested that LPMO inactivation leads to release of copper ions from the active site, which may cause fast degradation of ascorbic acid (Stepnov et al., 2022). Free ions of copper (and other transition metals) have also been extensively studied for their role in the metal-catalyzed decomposition of H_2O_2, leading to formation of hydroxyl and superoxide radicals (Pham et al.,

2013). It is possible that observed degradation of Glc4gemGlc reflects a situation where free copper has been released and H_2O_2 is degraded to radicals.

The results shown in **Figure 1** are in agreement with previous work where H_2O_2 was supplied at rates between 90–600 µM/h in saccharification reactions with 10% (w/v) Avicel and Cellic CTec2 (Müller et al., 2018). In both studies a feed rate of 80-90 µM/h led to the highest glucan conversion after 48 h, and reactions with this feed rate clearly outperformed aerobic control reactions

Figure 1 also shows that reactions with the LPMO-poor CelBG cocktail or with the Cellic CTec2 (CC2) cocktail under conditions that prevent LPMO action (i.e., anoxic, no H_2O_2 feeding) gave the lowest glucose yields and low levels of LPMO products. It is noteworthy that under anoxic conditions, the LPMO-poor cocktail CelBG outperformed CC2 (**Figure 1A**). This may be due in part to the higher proportion of cellulases in the LPMO-poor CelBG cocktail as compared with CC2, which may contain up to 20% (w/w, protein basis) LPMOs that are inactive under these conditions (Hu et al., 2015; Müller et al., 2015). In the aerobic reaction, LPMO activity ceases after 16 h. This is due to depletion of ascorbic acid, which is needed for *in situ* generation of H_2O_2. Note that the concentration of Glc4gemGlc is stable for the rest of the incubation period, indicating that, in this case, LPMO activity likely ceased due to lack of reductant and not inactivation of the LPMOs.

The impact of supplying H_2O_2 on lactic acid yields in SHF of Avicel

Next, we assessed the impact of supplying H_2O_2 during saccharification and fermentation on lactic acid production and if differences in the yields could be linked to LPMO activity. As fermentative strain, we used *B. coagulans*, which is able to grow and convert glucose to lactic

239 acid at 50 °C, the optimal temperature of the enzyme mixtures. This was done to decouple
240 temperature effects from potential LPMO effects. A fixed total process time of 48 h was selected
241 to be able to compare lactic acid production to the achieved total sugar release in **Figure 1**. The
242 SHF setup involved a 24 h saccharification phase followed by a 24 h fermentation phase during
243 which enzyme activity and, where applicable, H_2O_2 feeding, continued.

244 As in the saccharification experiments (**Figure 1**), the highest glucose concentrations and
245 LPMO product levels in the SHF saccharification phase (at 24 h, before microbial inoculation)
246 were achieved in the reactions with H_2O_2 feeding (**Figure 2A, C**). After the addition of the
247 microbial strain at 24 h, both glucose and Glc4gemGlc levels declined (**Figure 2A, C**),
248 suggesting their simultaneous consumption by *B. coagulans*. Earlier, Müller et al. (Müller et al.,
249 2017) had concluded that *B. coagulans* takes up Glc4gemGlc, using an SHF setup under aerobic
250 conditions. It must be noted though that the decrease in Glc4gemGlc levels may also be in part
251 due to the abovementioned instability of this compound, at least for the reaction with highest
252 H_2O_2 feed (Müller et al., 2018). However, the SSF experiments discussed below strongly suggest
253 that the LPMO product is consumed by *B. coagulans*.

254 **Figure 2** reveals signs of oxidative stress on the fermentative organism in the SHF setup for
255 all reactions with added H_2O_2 or air. Firstly, after inoculation, the decrease in glucose and
256 Glc4gemGlc levels set in later (after 28 h) for the reaction with the highest (160 µM/h) H_2O_2
257 feed rate compared to reactions with lower (40 and 80 µM/h) H_2O_2 feed rates (after 24 and 26 h,
258 respectively; **Figure 2A, C**). Secondly, lactic acid accumulation was the slowest in the setup
259 with the highest (160 µM/h) H_2O_2 feed rate (**Figure 2E**). As discussed above, the LPMO product
260 accumulation curve for the 160 µM/h reaction in **Figure 1B** indicates accumulation of damaging
261 levels of H_2O_2. In a previous report, Kadić et al. (Kadić et al., 2021) estimated that H_2O_2 levels

in situations like this may reach hundreds of micromolar. Pulses of H_2O_2 at such concentrations are often used to cause oxidative stress in cellular models (Ransy et al., 2020). Thirdly, glucose consumption rate was the slowest during fermentation in the reactions with the highest (160 µM/h) H_2O_2 feed rate and was also retarded in the other reactions with H_2O_2 feeding and in the aerated reaction, compared to the two anoxic reactions with no H_2O_2 feeding (CC2 and CelBG) where all glucose was consumed prior to 48 h.

Taken together, these observations show a negative impact on *B. coagulans* of H_2O_2 accumulation and continuous aeration. To handle oxidative stress, bacteria, including *Bacillus* spp., encode catalases and superoxide dismutases that scavenge reactive oxygen species (converting H_2O_2 to water and O_2 and superoxide to H_2O_2, respectively) as part of their cellular defense mechanism (Dowds, 1994; Vassilyadi & Archibald, 1985). As oxidative stress exerted by H_2O_2 is mitigated by converting H_2O_2 to O_2 by catalases (Ransy et al., 2020), the similar negative impact of high levels of H_2O_2 (Ransy et al., 2020) and aeration (Payot et al., 1999; Vassilyadi & Archibald, 1985) on glucose consumption and lactic acid production (as observed in **Figure 2A,E**) is not surprising.

It must be noted that the continued feeding of H_2O_2 was needed in this hybrid setup, to ensure LPMO activity during the second 24 h of the SHF process, which, as demonstrated by **Figure 1**, is needed to obtain high degrees of saccharification. Of course, other SHF scenarios that would not expose the bacterium to H_2O_2 or O_2 are possible, but these would lead to extended processing times, for example a scenario with a 48 h saccharification step with H_2O_2 feeding, followed by an anaerobic fermentation step without H_2O_2 feeding.

While there was only little difference in the levels of lactic acid produced at 48 h, i.e. after 24 h fermentation in the SHF setup (**Figure 2E**; **Table 2**), it is worth noting the considerable

amounts of residual glucose (and Glc4gemGlc) at 48 h (**Figure 2A, C**) in all reactions containing O_2 or H_2O_2. Thus, these reactions, and in particular the reactions with H_2O_2 feed at 80 and 160 µM/h, have a higher potential for lactic acid production that would probably be achieved by extending the fermentation period. If we assume full conversion of the residual glucose in these conditions, final lactic acid concentrations of 47.9 and 49.6 g/L could be reached in SHF reactions with H_2O_2 feed at 80 and 160 µM/h, respectively. For the 80 µM/h SHF reaction this is clearly lower than the glucose release demonstrated in the saccharification experiment which reached 72 g/L glucose. One reason could be lower LPMO activity in the last 24 h in the SHF setup due to consumption of H_2O_2 by the microbe.

Underpinning the impact of redox stress, glucose consumption (**Figure 2A**) and its concomitant conversion to lactic acid (**Figure 2E**) were the fastest (in the first 12 h of fermentation) in the anoxic setup with the LPMO-poor Celluclast–BG mixture (CelBG). This reaction, however, generated less glucose, and the final yield of lactic acid was lower than in the other reactions.

The impact of supplying H_2O_2 on lactic acid yields during SSF of Avicel

When adding *B. coagulans* at the same time as the enzyme cocktail, i.e., at t=0 h, in the SSF setup, glucose levels stayed close to zero, except for a small peak emerging during the first 12 h of the reaction (**Figure 2B**). This shows that, after 12 h, all solubilized glucose was fermented instantly and residual glucose levels after 48 h were below 0.5 g/L in all reactions (**Table 2**). Glc4gemGlc levels also stayed low (relative to the SHF reactions; compare **Figures 2C and 2D**) but remained clearly above zero for several of the reactions (**Figure 2D**). These low LPMO product levels indicate uptake by the bacterium, although we cannot exclude that they are due, in

307 part, to low LPMO activity during SSF even with H_2O_2 supply. While LPMO activity could not
308 be quantified by measuring the formation of Glc4gemGlc, lactic acid yields clearly revealed the
309 positive impact of H_2O_2 supply on the overall conversion yield. The highest lactic acid yield of
310 52.2 g/L was achieved in the SSF setup with 80 µM/h H_2O_2 feed, which is the feed rate that led
311 to the highest glucose yield in the control saccharification experiments (**Figure 1A**).
312 **Figure 1** shows that aeration clearly improves LPMO activity and saccharification yields. On
313 the other hand, **Figures 2E,F** show a negative impact of aeration on lactic acid production, likely
314 due to less efficient conversion of glucose to lactic acid by *B. coagulans*. Even though higher
315 glucose levels were reached when supplying air to the reaction with Cellic CTec2 (CC2_Air)
316 compared to the anoxic equivalent (CC2) (**Figure 1A**), the lactic acid yields were similar (SHF;
317 **Figure 2E**) or lower (SSF; **Figure 2F**) under aerobic condition. Our results are in agreement
318 with previous reports on the negative effect of aeration on lactic acid yields, partly due to co-
319 production of other organic acids (Müller et al., 2017; Payot et al., 1999). Previous SSF studies
320 with LPMO-containing cellulase cocktails, which did not consider the role of H_2O_2, have shown
321 that under aerobic conditions, SHF works better than SSF (Cannella & Jørgensen, 2014; Müller
322 et al., 2017). Negative effects of H_2O_2 on the fermenting strain were less apparent in the SSF
323 than in the SHF setup, especially with the highest H_2O_2 feed rate (CC2_160; **Figure 2F**). Unlike
324 the SHF setups, the SSF setups with H_2O_2 feed showed neither an initial delay in glucose
325 conversion nor an apparent slower consumption of glucose. One reason for this may be the lower
326 levels of oxidative stress at the time of inoculation in the SSF process setup compared with SHF.
327 For SSF, the fermentative strain is added in the beginning of the reaction (t=0 h) when most
328 H_2O_2 is being consumed by active LPMOs and H_2O_2 levels remain low, even in the setup with
329 the highest H_2O_2 feed rate (see the work by Kadić et al. (2021)). In the SHF setup with 160 µM/h

330 H_2O_2 feed, on the other hand, the H_2O_2 level may be in the range of hundreds of micromolar at
331 the time of inoculation (t=24 h), as outlined above.
332 From the above, it is clear that, for fuelling LPMO reactions during SSF, it is better to
333 administer H_2O_2 than O_2. While the continuous removal of LPMO products during the SSF
334 prevented quantification of LPMO activity, the importance of these enzymes seems evident,
335 based on the data presented in **Figure 1** and, more so, the positive impact of the "right" level of
336 H_2O_2 feeding on lactic acid production in the SSF (**Figure 2F**). Importantly, the anoxic SSF
337 reaction with LPMO-poor CelBG produced much less lactic acid than the reactions with Cellic
338 CTec2 (CC2).
339 It is noteworthy that the lactic acid production levels in the SSF experiments did not always
340 correlate with the degree of cellulose saccharification after 48 h (depicted in **Figure 1**), as
341 already mentioned above for the aerated reaction with Cellic CTec2. Such discrepancy is,
342 generally, not surprising since the enzymatic degradation reaction, in particular its redox part,
343 and bacterial fermentation may affect each other in multiple ways. For example, the bacteria may
344 remove H_2O_2 through their catalases or secrete oxidases that generate H_2O_2 in aerated reactions.
345 Bacterial metabolisms could also lead to release of redox-active compounds that react with H_2O_2
346 or the LPMO. Bacterial metabolism may be affected by the presence of O_2 and H_2O_2. In aerated
347 cultures, accumulation of acetate has been observed previously (Müller et al., 2017). In this
348 study, we did not detect formation of acetate in any of the reactions. The reaction with Cellic
349 CTec2 under anoxic conditions without H_2O_2 feeding (CC2; **Figure 2F**) provides the largest
350 discrepancy. This reaction was among the least efficient when looking at saccharification only
351 and displayed low LPMO product levels (**Figure 1A**). Nevertheless, in the anoxic SSF reaction
352 without H_2O_2 feeding (CC2), lactic acid concentrations reached similar levels 40.0 g/L) as in

reactions with low (CC2_40; 38.2 g/L lactic acid) and high (CC2_160; 39.2 g/L lactic acid) H_2O_2 feed rates (**Figure 2F**; **Table 2**). The most obvious explanation for this observation is that the conversion of glucose to lactic acid is most efficient in a fully anoxic environment in the absence of H_2O_2.

Despite possible "mixed" effects of feeding H_2O_2, the overall positive impact of driving oxidative cellulose saccharification with H_2O_2 is striking for the setup with the 80 μM/h H_2O_2 feed. This reaction setup yielded significantly higher amounts of lactic acid (CC2_80; 52.2 g/L) at 48 h of SSF compared to any of the other reactions.

Assuming that one gram of glucose yields one gram of lactic acid under optimal conditions (Abdel-Rahman et al., 2013), in this study, the final yields of lactic acid in the SSF processes varied from 24-47% relative to available Avicel. It should be noted that Avicel is a model substrate which is challenging to fully degrade. A previous study has shown final lactic acid yields corresponding to ca. 60 and 70% cellulose utilization for an aerobic SSF and SHF setup, respectively, with washed steam-exploded birch (Müller et al., 2017). In general, lactic acid yields in the range of 86-92% can be reached from pure glucose and xylose for *B. coagulans* DSM2314 (van der Pol et al., 2016). Thus, while some conditions showed promising lactic acid yields, further analyses of the factors limiting lactic acid production is needed. The present proof-of-concept study shows ways of harnessing LPMO activity in SSF processes and can be used as a basis for further process optimization.

When further optimizing the process, it is important to also take into account that the accumulation of lactic acid may hinder enzymatic activity towards the end of the reaction. It has been previously established that glucan conversion by *Trichoderma reesei* cellulases (Iyer & Lee, 1999) and the cellulase cocktail Genencor GC220 (van der Pol et al., 2016) is negatively

impacted by the presence of lactic acid at concentrations above 30 and 50 g/L, respectively. Thus, continuous removal of lactic acid during fermentation may be considered and, obviously, care should be given to avoiding acidification. Another consideration may be to engineer catalase-deficient *Bacillus* spp. (such as the *Bacillus* strains (Vassilyadi & Archibald, 1985) in order to limit H_2O_2 uptake or decomposition by the fermenting strain.

Concluding remarks

The prospect of being able to promote LPMO activity during anoxic fermentation reactions by feeding H_2O_2 opens up new avenues for using one-pot SSF approaches in the valorization of lignocellulosic biomass. Both the present (**Figure 1**) and previous studies leave no doubt that LPMO activity is important for efficient saccharification of cellulose, and it has been demonstrated that LPMOs do not work in anaerobic environments. We show here that it is fully possible to maintain both LPMO activity and microbial production under anoxic conditions, by continuously feeding reactions with low amounts of H_2O_2. While our studies do not reveal the exact fate of the added H_2O_2, which may be used by the LPMO or converted by the redox house-keeping machinery of the fermenting microbe, it is clear that in some of the reaction setups explored above, H_2O_2 levels were sufficient to drive the LPMO reaction while not harming microbial fermentation.

Despite the signs of redox stress seen in the SHF experiments, likely due to overfeeding with H_2O_2 in the fermentation phase, true SSF experiments with similar H_2O_2 feeding worked well and did not show signs of growth inhibition or lack of glucose utilization. Apparently, in the SSF setup, with appropriate H_2O_2 feeding, the reaction was well-balanced. It is not straightforward to

explain why the SSF worked so well. It is possible that the bacteria perform better when being fed gradually with glucose, as in the SSF. Also, exposure of the bacteria to H_2O_2 will vary between the two regimes because the levels of H_2O_2 that are not consumed in productive reactions catalyzed by the LPMO will likely vary as the substrate is degraded. Finally, the bacteria may affect LPMO action and stability.

More research is needed to optimize these SSF processes, and only a few approaches were assessed in the present proof-of-concept study. The SHF approach could be explored further and optimized. Other H_2O_2-feeding regimes, such as varying the feed rate over time, could be explored in both SHF and SSF. It must be noted that for the SSF processes, lactic acid yields were in the order of 24-47% of the theoretical maximum based on the initial Avicel concentration. The microcrystalline substrate Avicel is a model substrate and not easily hydrolysable. Previously, higher lactic acid yields have been reached on pretreated lignocellulosic substrates where the cellulose is more accessible. There is therefore potential to reach higher yields with this novel SSF setup using industrially relevant substrates. Moreover, the present results clearly show the feasibility of harnessing the power of LPMOs in such processes.

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Tables

Table 1. Different process setups for saccharification and fermentation of Avicel. Reactions with 1 L total volume contained 10% (w/v) Avicel in 50 mM sodium acetate buffer at pH 5.5. Enzymes (4 mg/g DM Cellic CTec2, denoted as CC2, or Celluclast–BG mixture, denoted as CelBG) were added at t=0 h, followed immediately by adding inoculum and/or starting H_2O_2 feed, as indicated. All reactions were run in duplicate.

Process ID*	**Enzyme cocktail**	**H_2O_2 feed (µM/h)**	**Strain addition**	**Atmosphere****
Sac_CelBG	Celluclast + BG	0	No	N_2
Sac_CC2	Cellic CTec2	0	No	N_2
Sac_CC2_Air	Cellic CTec2	0	No	Air
Sac_CC2_40	Cellic CTec2	40	No	N_2
Sac_CC2_80	Cellic CTec2	80	No	N_2
Sac_CC2_160	Cellic CTec2	160	No	N_2
SHF_CelBG	Celluclast + BG	0	*B. coagulans*, 24 h	N_2
SHF_CC2	Cellic CTec2	0	*B. coagulans*, 24 h	N_2
SHF_CC2_Air	Cellic CTec2	0	*B. coagulans*, 24 h	Air
SHF_CC2_40	Cellic CTec2	40	*B. coagulans*, 24 h	N_2
SHF_CC2_80	Cellic CTec2	80	*B. coagulans*, 24 h	N_2
SHF_CC2_160	Cellic CTec2	160	*B. coagulans*, 24 h	N_2
SSF_CelBG	Celluclast + BG	0	*B. coagulans*, 0 h	N_2
SSF_CC2	Cellic CTec2	0	*B. coagulans*, 0 h	N_2
SSF_CC2_Air	Cellic CTec2	0	*B. coagulans*, 0 h	Air
SSF_CC2_40	Cellic CTec2	40	*B. coagulans*, 0 h	N_2
SSF_CC2_80	Cellic CTec2	80	*B. coagulans*, 0 h	N_2
SSF_CC2_160	Cellic CTec2	160	*B. coagulans*, 0 h	N_2

* Sac, Saccharification; SHF, Separate Hydrolysis and Fermentation; SSF, Simultaneous Saccharification and Fermentation; BG, beta-glucosidase. N_2 represents continuous sparging of N_2 gas at 100 mL/min, Air represents aerobic headspace.

**Air in headspace or N_2 sparging.

Table 2. Glucose and lactic acid concentrations at the endpoint (48 h) in the various SHF and SSF reactions detailed in Table 1. In brief, Cellic CTec2 (CC2) or a Celluclast–BG mixture (CelBG) was used for glucose generation, and glucose was converted to lactic acid with *B. coagulans.*

	Glucose concentration (g/L)			**Lactic acid concentration (g/L)**	
	Sac	**SHF**	**SSF**	**SHF**	**SSF**
CelBG	39.6 ± 0.4	0.3 ± 0.1	0.1 ± 0.0	31.4 ± 0.5	26.7 ± 0.9
CC2	30.9 ± 5.2	0.2 ± 0.1	0.2 ± 0.0	38.6 ± 1.0	40.0 ± 0.4
CC2_Air	43.4 ± 9.1	1.7 ± 2.3	0.2 ± 0.0	39.6 ± 5.8	34.4 ± 8.2
CC2_40	49.8 ± 0.4	2.4 ± 0.1	0.1 ± 0.0	39.4 ± 6.0	38.2 ± 1.5
CC2_80	72.5 ± 1.4	6.4 ± 1.2	0.4 ± 0.2	41.5 ± 0.1	52.2 ± 1.7
CC2_160	54.6 ± 0.3	13.5 ± 3.9	0.0 ± 0.1	36.1 ± 4.7	39.2 ± 0.6

Figures

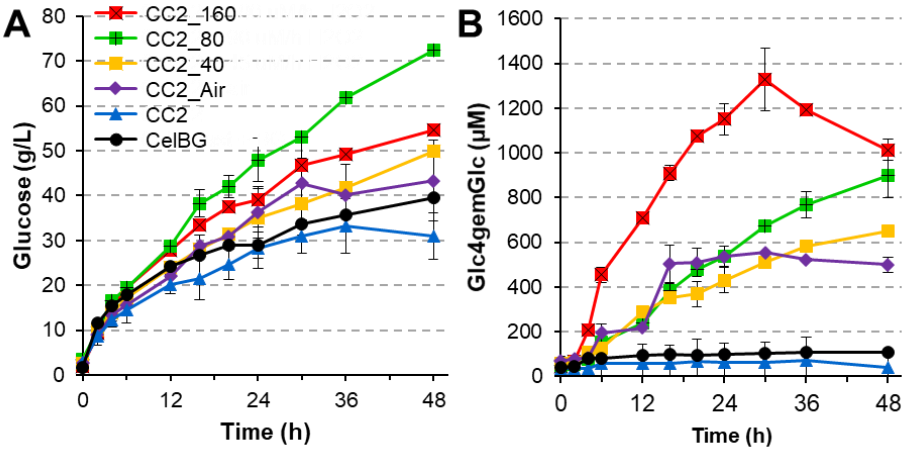
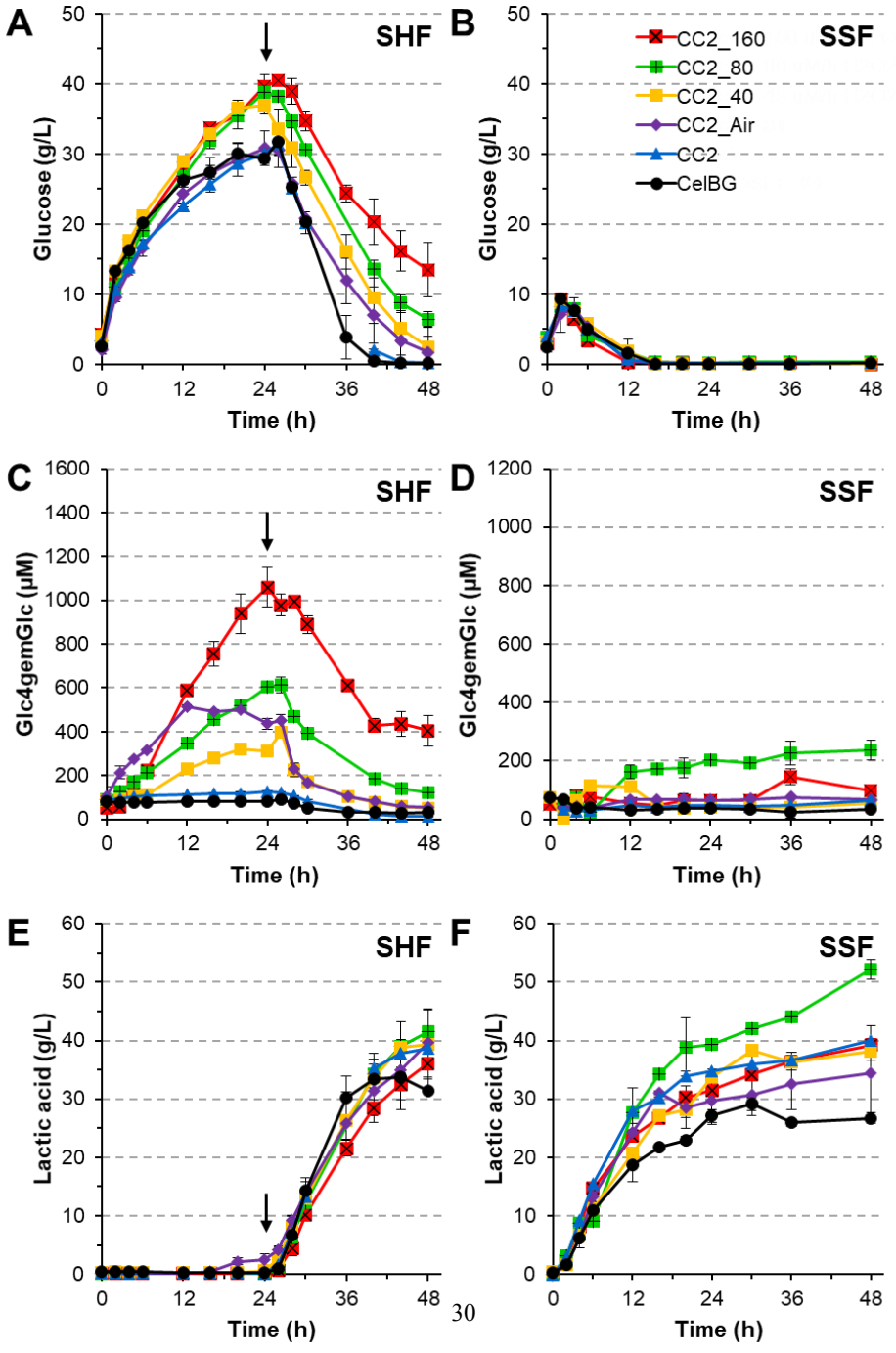


Figure 1. Glucose release (A) and LPMO product levels (B) during saccharification of cellulose. Avicel (10%, w/v) was incubated with 4 mg/g substrate of cellulase cocktail, either Cellic CTec2 (CC2) or a Celluclast–BG mixture (CelBG) in 50 mM Na-acetate buffer (pH 5.5) at 50 °C with 1 mM AscA as reducing agent. All reactions were run in nitrogen atmosphere except CC2_Air, which was aerated. Some reactions were fed with H_2O_2 at 40 (orange), 80 (green), or 160 µM/h (red) feed rate. The LPMOs in Cellic CTec2 are predominantly C4-oxidizing and the cellulases in Cellic CTec2 convert longer LPMO products to the oxidized dimer, which explains why Glc4gemGlc levels were used as proxy for LPMO activity. Error bars displayed in figures are based on two biologically independent replicates.


A
SHF
Glucose (g/L)
Time (h)
B
SSF
CC2_160
CC2_80
CC2_40
CC2_Air
CC2
CelBG
C
SHF
Glc4gemGlc (µM)
D
SSF
E
SHF
Lactic acid (g/L)
F
SSF

590

Figure 2. Glucose (A,B), Glc4gemGlc (C,D), and lactic acid (E,F) levels in 1 L SHF (A,C,E) and SSF (B,D,F) of cellulose. Avicel (10%, w/v) was incubated with 4 mg/g substrate cellulase cocktail, either Cellic CTec2 (CC2) or a Celluclast–BG mixture (CelBG) in 50 mM Na-acetate buffer (pH 5.5) at 50 °C with 1 mM AscA as reducing agent. All reactions were run in nitrogen atmosphere, except CC2_Air, which was aerated. Some reactions were fed with H_2O_2 at 45 (orange), 90 (green), or 160 µM/h (red) feed rate for the full duration of the experiment. A *B. coagulans* preculture was added at 24 h (for SHF; panels **A,C,E** – indicated by arrows) or at 0 h (for SSF; panels **B,D,F**) with a starting OD_{600} of 0.5. Error bars displayed in figures are based on two biologically independent replicates.

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