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*
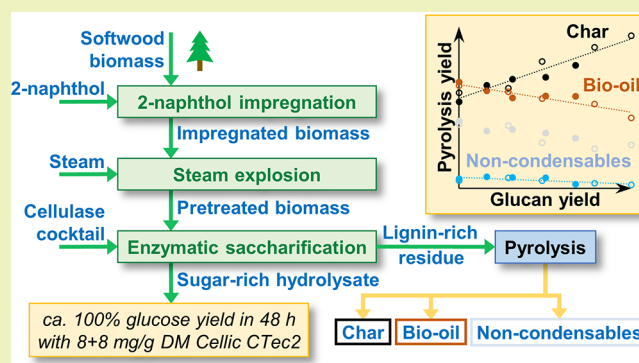
INTRODUCTION

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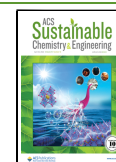
condensation[9,10,16] as well as improve the enzymatic digestibility of cellulose in steam-pretreated feedstocks.[11]

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 (SmAA10A) 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.

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 SmGH20A 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
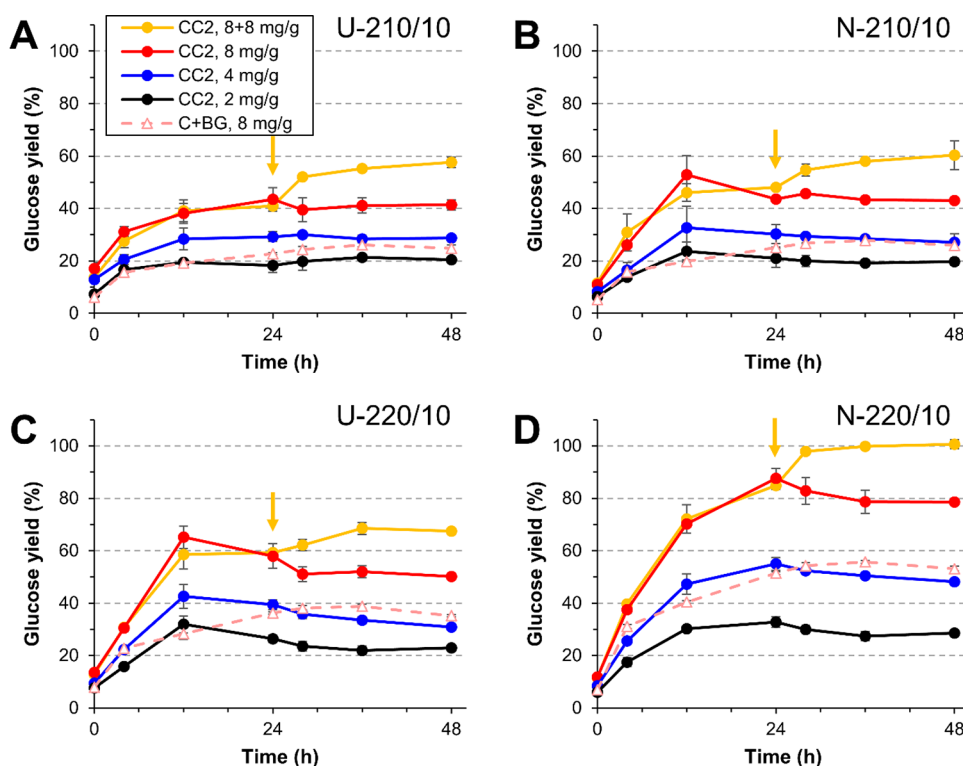
Figure 1. Glucose yields during enzymatic saccharification of pretreated Norway spruce. Norway spruce pretreated at 210 °C (A, B) or 220 °C (C, D) without impregnation (A, C) and with 2-naphthol impregnation (B, D) was subjected to saccharification with Cellic CTec2 (CC2) at various enzyme loadings or 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
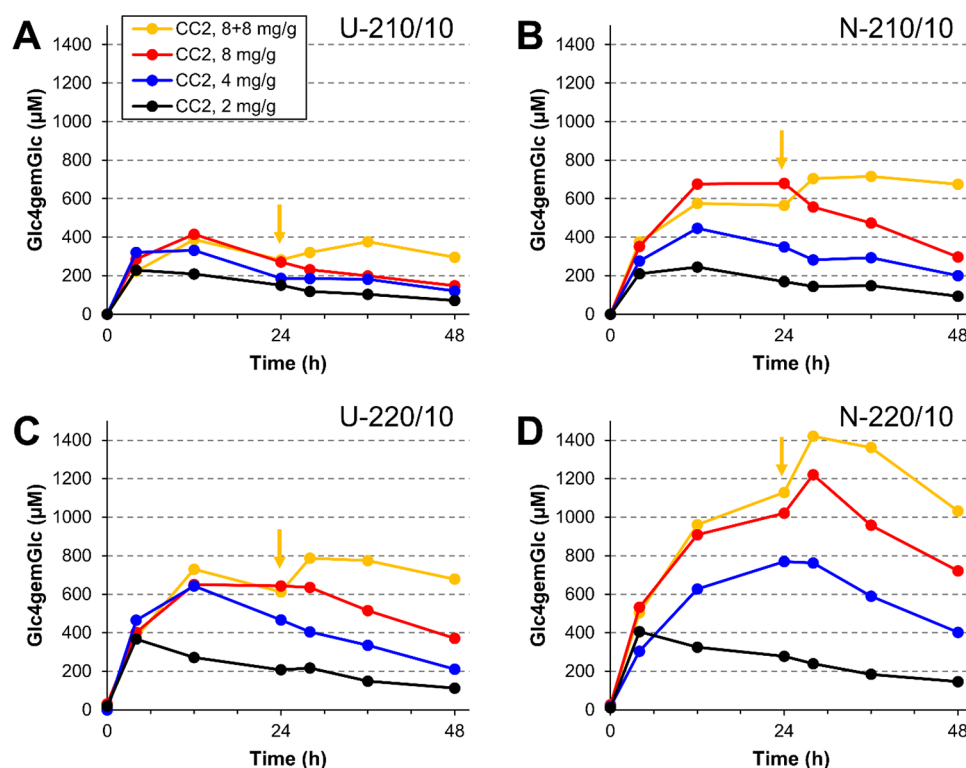
Figure 2. Glc4gemGlc (C4-oxidized cellobiose) levels during enzymatic saccharification of pretreated Norway spruce. Norway spruce pretreated at 210 °C (A, B) or 220 °C (C, D) without impregnation (A, C) and with 2-naphthol impregnation (B, D) was subjected to saccharification with Cellic CTec2 at various 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
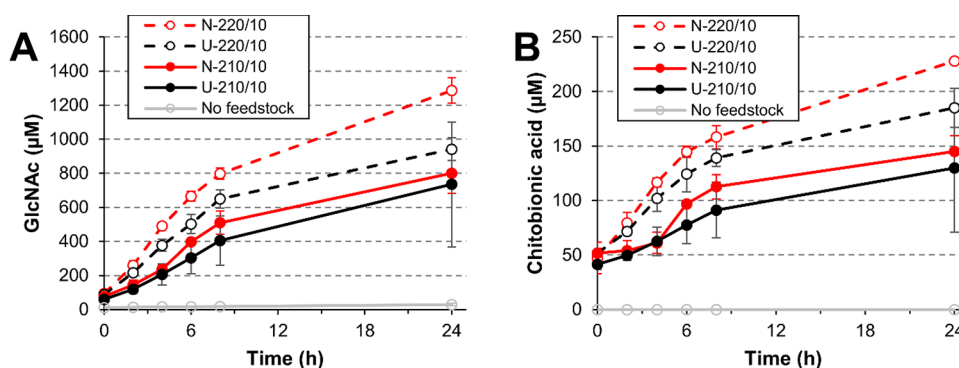
Figure 3. Comparison of the abilities of differently pretreated Norway spruce feedstocks to drive an LPMO reaction. Reactions containing 1% (w/v) β-chitin and 1 μM CBP21 in 50 mM Bis−Tris/HCl buffer (pH 6.5) were supplemented with 1% (w/v) of one of four differently pretreated spruce feedstocks, as indicated 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 (SmGH20A) 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 (SmAA10A, 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
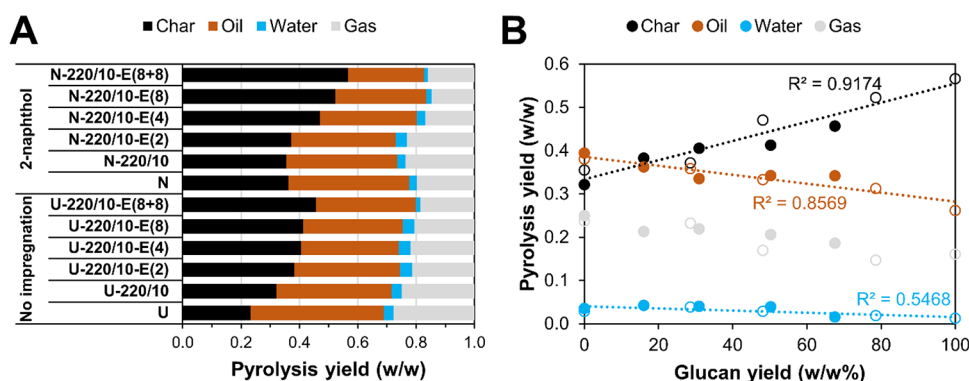
Figure 4. Product formation upon pyrolysis of selected spruce fractions. The graphs show yields of various pyrolysis products as a weight ratio of the total amount of products for native spruce without (U) or with (N) 2-naphthol impregnation, steam-exploded spruce without (U-220/10) or with (N-220/10) 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.
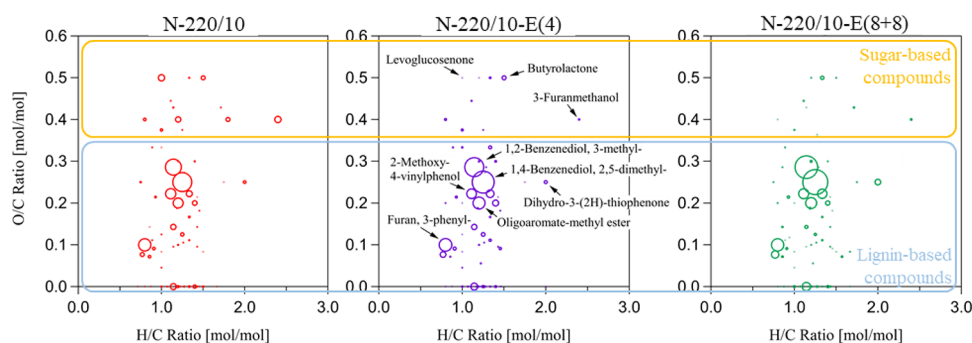

Figure 5. Van Krevelen plots for pyrolysis liquids obtained upon pyrolysis of selected spruce fractions with the dominant compounds assigned. The figure shows van Krevelen diagrams for pyrolysis liquids derived from a 2-naphthol-impregnated steam-exploded feedstock without (N-220/10) or with ca. 50% (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

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.

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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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