Supporting information for

Lignin repolymerisation in spruce autohydrolysis pretreatment increases cellulase deactivation

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Overview of pretreatment experiments

Table S1 Overview of all pretreatment experiments. Reactor charge: 2.5g spruce, 39.2g H_20

Document section	Additive			t @ 210 °C / min	$logR_0$***
	Compound	mg	mol/mol lignin C_9 unit*		
3.1	2-naphthol	118.5	0.205	0**	4.5
3.1	2-naphthol	118.5	0.205	10	4.7
3.1	2-naphthol	118.5	0.205	20	4.8
3.1	2-naphthol	118.5	0.205	60	5.1
3.1	2-naphthol	118.5	0.205	120	5.4
3.1	2-naphthol	118.5	0.205	240	5.6
3.1	resorcinol	45.2	0.103	0**	4.5
3.1	resorcinol	45.2	0.103	10	4.7
3.1	resorcinol	45.2	0.103	20	4.8
3.1	resorcinol	45.2	0.103	60	5.1
3.1	resorcinol	45.2	0.103	120	5.4
3.1	resorcinol	45.2	0.103	240	5.6
3.1	-	-	-	0**	4.5
3.1	-	-	-	10	4.7
3.1	-	-	-	20	4.8
3.1	-	-	-	60	5.1
3.1	-	-	-	120	5.4
3.1	-	-	-	240	5.6
3.2	-	-	-	120	5.4
3.2	2-naphthol	25	0.051	120	5.4
3.2	2-naphthol	50	0.103	120	5.4
3.2	2-naphthol	100	0.205	120	5.4
3.2	2-naphthol	200	0.413	120	5.4
3.2	2-naphthol	400	0.823	120	5.4

*assumed molecular weight of lignin C_9 unit: 185g/mol
**reactor heated up to pretreatment temperature and cooled down immediately
***as defined in (Eq. 4)

Experimental details: calculation of pretreatment severity

In order to account for the heat-up phase in the pretreatment severity calculation, two severities were defined. The "base" pretreatment severity R_p is defined as (Eq. **1**).

$$R_p = t_p \cdot e^{\frac{T_p - 100}{14.75}} \quad \textbf{[1]}$$

where t_p is the pretreatment time in minutes and T_p the pretreatment temperature in degrees Celsius[1]. Additionally, a "heat-up" severity R_h was defined as (Eq. **2**).

$$R_h = \int_{t_h=0}^{t_h=12} t_h \cdot e^{\frac{T_h(t_h) - 100}{14.75}} dt \quad \textbf{[2]}$$

where t_h is the elapsed heating time in minutes and T_h the corresponding temperature in degrees Celsius. Approximating a linear heating behaviour from 100 to 210 °C, T_h can be expressed as:

$$T_h = 100°C + \frac{110°C}{12min} \cdot t_h \quad \textbf{[3]}$$

The total severity R_0 was then defined as the sum of the base and the heat-up severity:

$$R_0 = R_p + R_h \quad \textbf{[4]}$$

The quenching of the reactors below 100 °C was not taken into account for the severity calculations.

Experimental details: downscaled analysis of biomass and liquor from pretreatment

It has previously been shown that downscaling of the standard NREL routine[2] for biomass analysis by a factor of 100 is possible while still yielding accurate data[3]. Owing to the small amount of biomass used in the pretreatments and to speed up the analysis, the NREL method was downscaled by a factor of 56 and several procedural steps modified, as reported in the following:

5.35±0.3 mg of dried biomass were weighed in a 2 ml centrifuge tube, having recorded the weight of the empty tube. For the first hydrolysis step in a water bath, 53.6 µL of 72%w/w sulphuric acid were added and the slurry was mixed using a vortex shaker. For the second hydrolysis step, the samples were diluted by adding 1.5 ml of water and the tubes placed in a vial rack. The rack was clamped between two steel plates to ensure sealing of the tubes. The sandwich was clamped together using bolts in each corner of the plates together with spring washers and wing nuts. After autoclaving, 0.5 ml liquid of each sample were transferred to a new 1.5 ml centrifuge tube, neutralized with $CaCO_3$, centrifuged and 300 µL liquid were taken for HPLC analysis of the carbohydrates. ASL content was determined using a PerkinElmer Lambda 35 UV/VIS spectrophotometer at a wavelength of 240 nm. AIL content was determined as follows: the liquid remaining in the autoclaved tubes after the two previous analyses was removed with a micropipette and the solids washed three times by resuspending with 1.5 ml of DI water and centrifugation. The samples were dried at 105 °C for 36 h and reweighed after cooling down for at least 1 hour at room temperature. The mass of the remaining AIL (including ash) was then found by the difference to the weight of the empty tube. All biomass analyses were done in triplicate and single standard deviations are reported with the mean in this work.

Analysis of sugars and byproducts in the pretreatment liquor was done as specified by Sluiter[4], similarly downscaled as the biomass compositional analysis. 1.5 ml of filtrate were added to a 2 ml centrifuge tube, acidified with 53.6 µL of 72%w/w H_2SO_4 and autoclaved, neutralized and analysed for sugar concentration like the biomass samples. Analyses were done in duplicate and single standard deviations are reported with the mean in this work.

Experimental details: enhancing softwood digestibility (section 3.1)

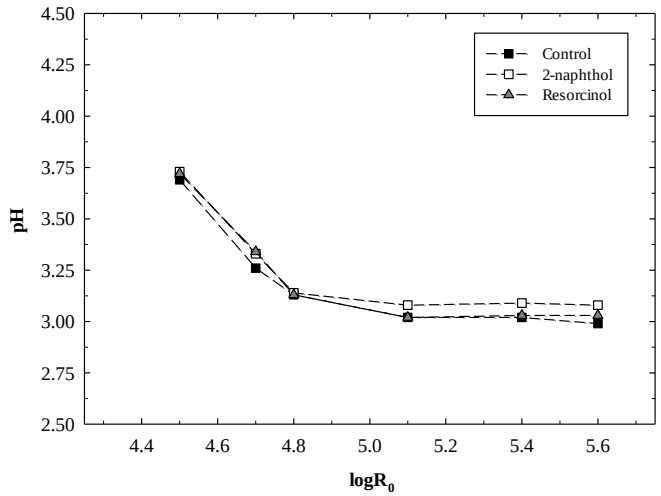


Fig. S1 pH of pretreatment liquor after pretreatment with 2-naphthol, resorcinol and no additive (control) at different severities.

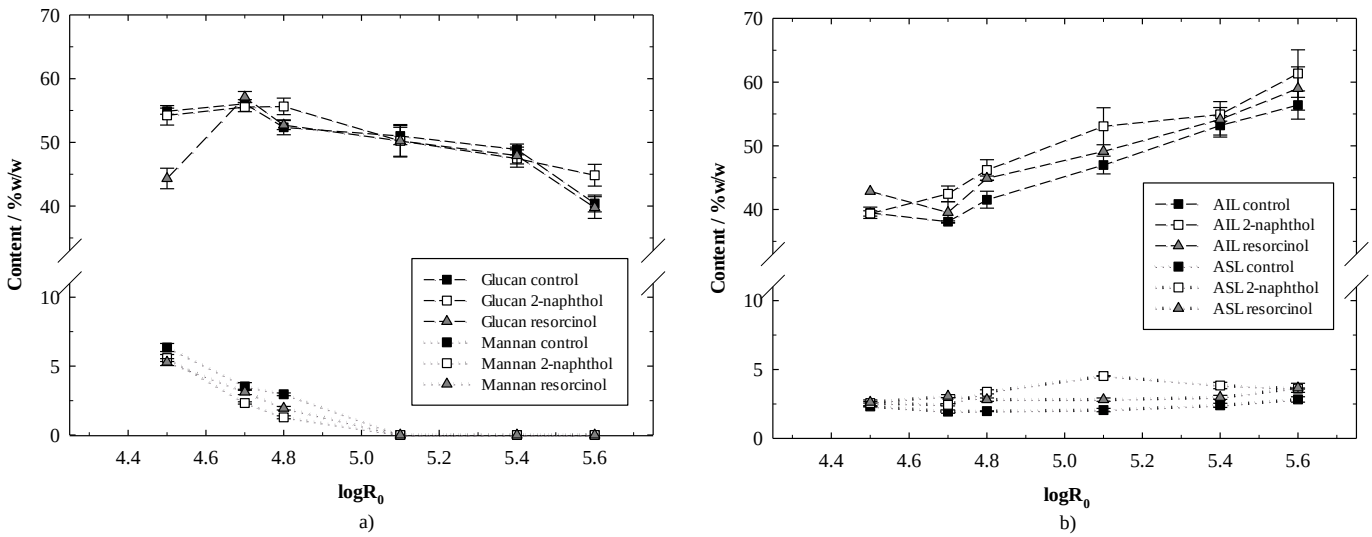
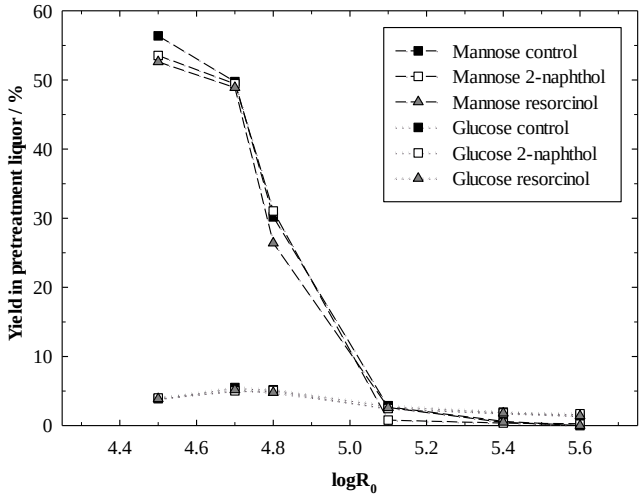


Fig. S2 Carbohydrate (a) and lignin (b) contents of spruce pretreated at different severities with 2-naphthol, resorcinol and without additive (control). Mannan represents total hemicellulose content. AIL: acid insoluble lignin, ASL: acid soluble lignin.

Sugar yields



During pretreatment, dissolved pentoses can degrade further to furfural[5] and hexoses to hydroxymethylfurfural (HMF)[6]. Hemi-cellulosic sugars were exposed to fast degradation due to their fast dissolution. Glucose from cellulose is set free more gradually, hence there was always some glucose present in the liquid, even at high severities. The glucose concentration exhibits a maximum at a severity of 4.7, possibly as a result of amorphous parts of cellulose that were dissolved faster or due to glucose being set free from hemicellulose.

Fig. S3 Yields of sugars dissolved in the pretreatment liquor as a function of pretreatment severity. Mannose represents sum of hemicellulosic sugars. Error bars not shown; all $\sigma < 0.23\%$.

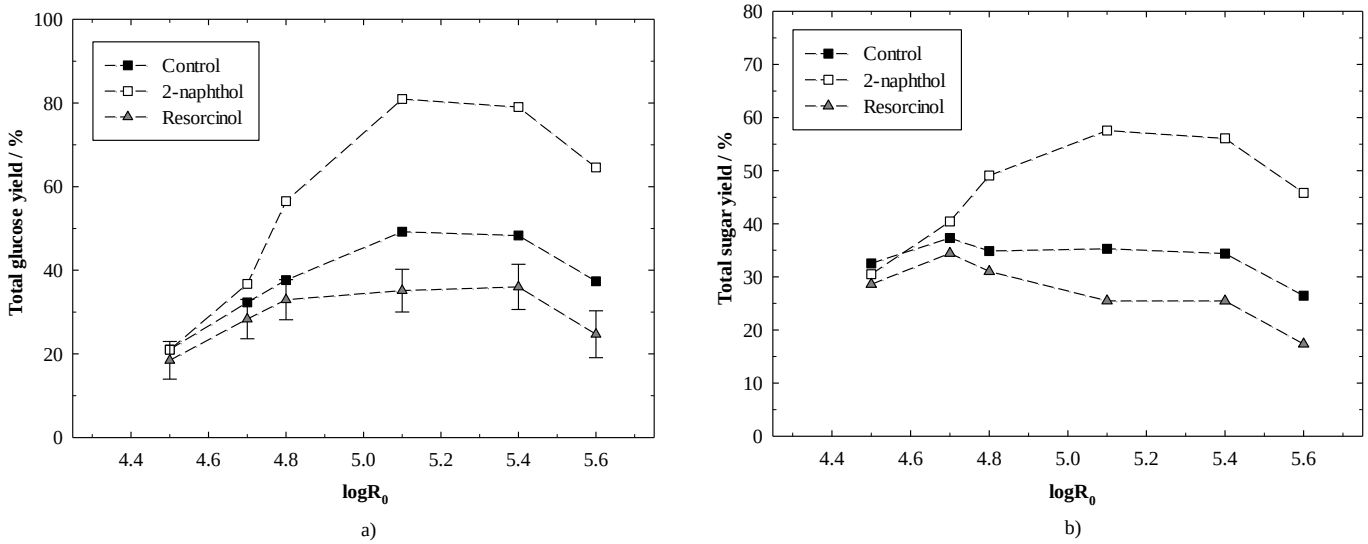


Fig. S4 Total glucose yield from enzymatic hydrolysis and pretreatment liquor (a) and total sugar yield from enzymatic hydrolysis and pretreatment liquor (b) as a function of pretreatment severity and additives.

2-naphthol conversion – In order to determine the conversion of 2-naphthol in the pretreatment, the concentration of not reacted 2-naphthol remaining in the liquor after pretreatment was estimated by extraction and gas chromatography/mass spectrometry (GC/MS) analysis.
10 ml of the pretreatment liquor were extracted three times with 5 ml chloroform and the organic phase was recovered. After three extractions no 2-naphthol could be detected in the aqueous phase anymore. 200 µl of a 1 mg/ml syringaldehyde solution were added as internal standard for quantification in the GC/MS analysis to the combined chloroform extracts. The GC/MS analysis was carried out as described elsewhere[7].

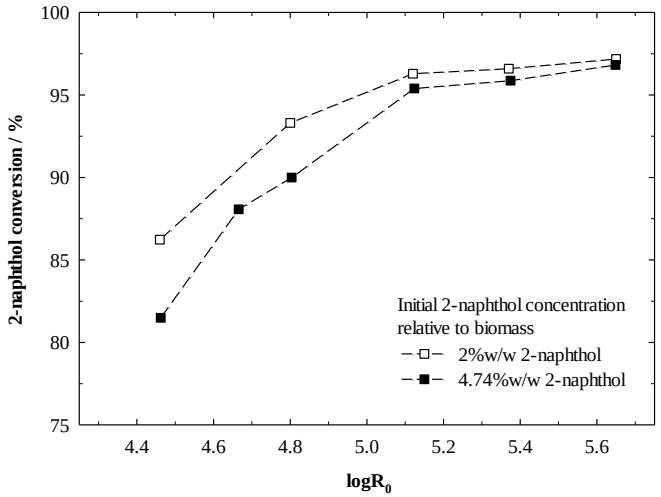


Fig. S5 2-naphthol conversion in the pretreatment as a function of pretreatment severity and initial 2-naphthol concentration.

Hydrolysis of pure cellulose in the presence of 2-naphthol – Hydrolysis of pure microcrystalline cellulose (Avicel PH-101, Fluka) in the presence of 2-naphthol was performed to study the influence of 2-naphthol on enzyme inhibition.
Hydrolysis was prepared in a similar manner as described in section 2.1. Samples of 25 ml were prepared with cellulose concentrations of 1 respectively 2.5%w/w and varying 2-napthtol concentrations between 0 and 0.356%w/w respectively 0 and 0.889%w/w. The enzyme dose was 15 FPU/g cellulose and the hydrolysis slurry was sampled 120 h after the start of the hydrolysis.

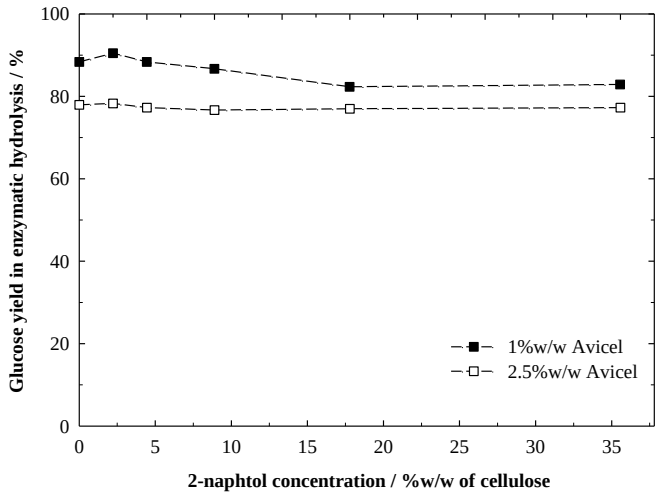


Fig. S6 Glucose yields in the enzymatic hydrolysis of Avicel (1 and 2.5%w/w) in the presence of different 2-naphthol concentrations. Hydrolysis conditions: 15 FPU/g cellulose, 120h.

Fermentation of glucose in the presence of 2-naphthol – Fermentation of glucose in the presence of 2-naphthol was performed to study the influence of 2-naphthol on yeast inhibition.
Saccharomyces cerevisiae D5A was grown under sterile conditions on YPD medium (10 g/l yeast extract, 20 g/l peptone and 10 g/l glucose) and varying concentrations of 2-naphthol between 0 and 0.4 g/l. Samples were prepared

in 100 ml serum bottles closed with rubber crimp caps and vented through 19 gauge needles. The samples were inoculated with 5% v/v of an overnight culture on YPD medium containing 50 g/l glucose, giving a total sample volume of 50 ml. The samples were incubated in a shaker (Multitron Pro; Infors-HT) with a shaking throw of 25 mm at 37 °C and 120 rpm. Samples were analysed for glucose and ethanol in the supernatant by HPLC and optical density of the slurry at 600 nm (DR6000, Hach-Lange). All fermentation experiments were carried out in triplicate and single standard deviations are reported with the mean.

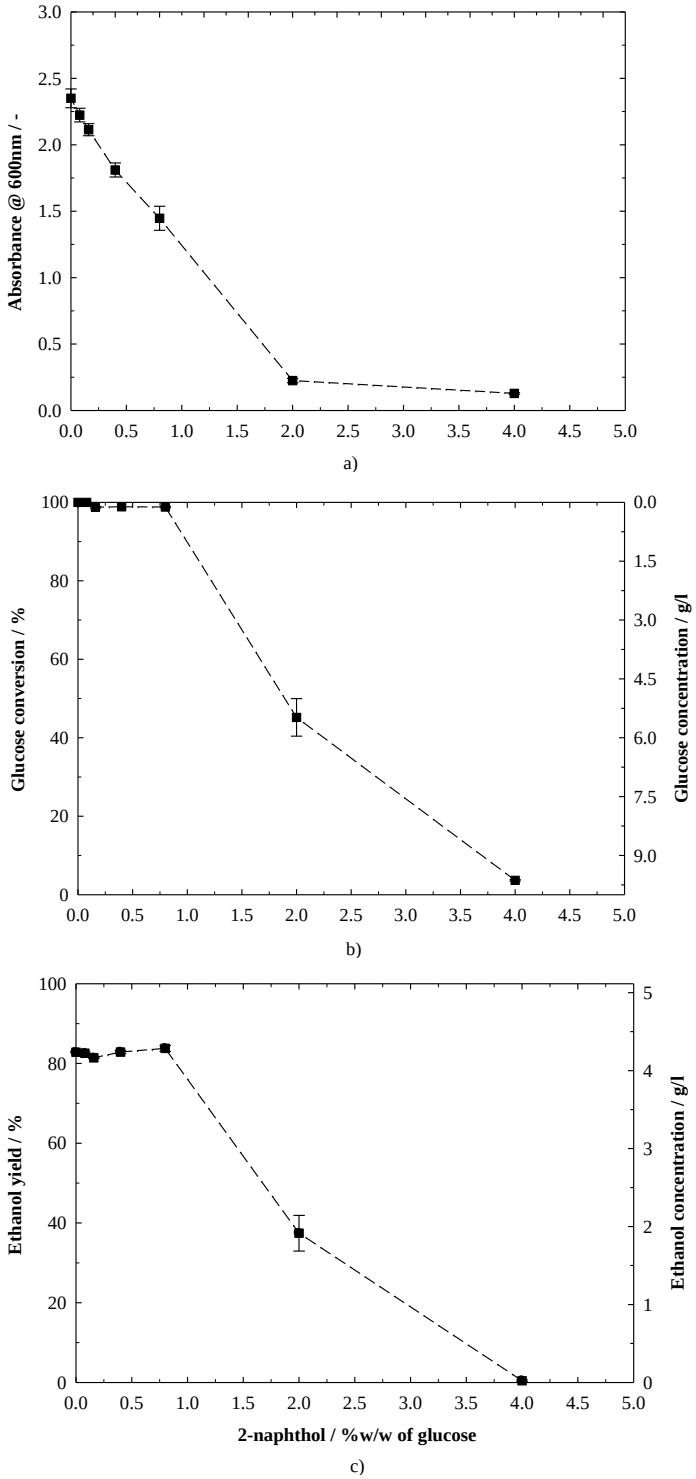


Fig. S7 Fermentation of glucose in the presence of different 2-naphthol concentrations. Shown are the absorbance of the fermentation broth at 600 nm (a), the glucose conversion (b) and the ethanol yield (c). Fermentation conditions: 10 g/l glucose, 24h.

Experimental details: effect of lignin repolymerisation on enzyme deactivation (section 3.2)

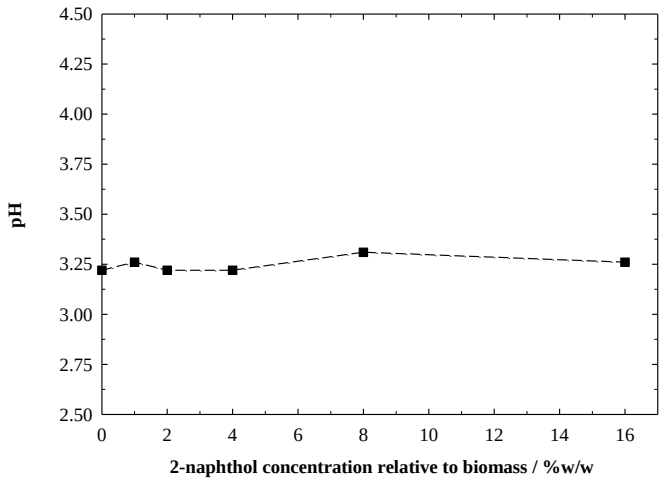


Fig. S8 pH of pretreatment liquor after pretreatment with different concentrations of 2-naphthol.

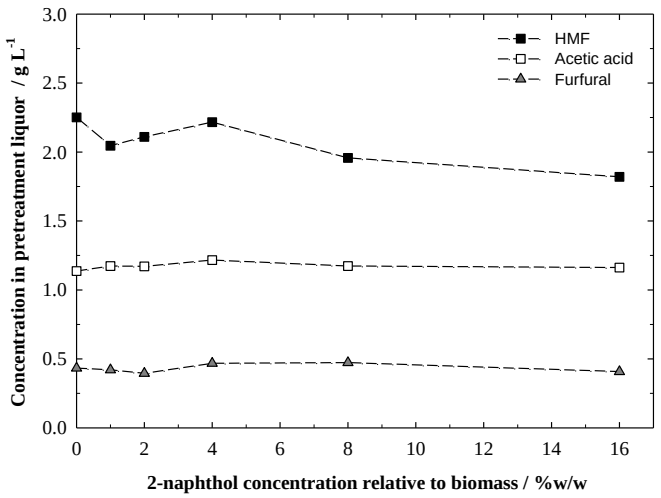


Fig. S9 Concentrations of acetic acid, HMF and furfural in the pretreatment liquors after pretreatment with different concentrations of 2-naphthol.

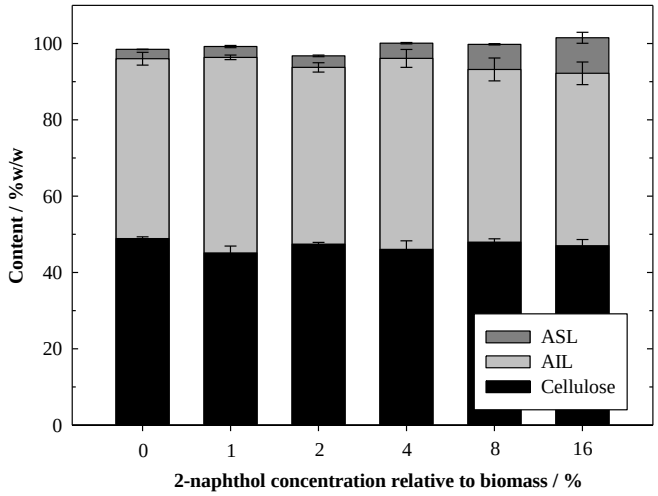


Fig. S10 Composition of spruce pretreated with different concentrations of 2-naphthol.

FTIR analysis - Fourier transform infrared (FTIR) spectra of the pretreated biomass samples were recorded with a PerkinElmer Spectrum BX spectrometer in transmission mode (Scan range: 4000-600 cm-1, number of scans: 64, resolution: 2.0 cm-1). Biomass samples were dried overnight under vacuum at 50 °C. Pellets were prepared similar as described by Faix[8] by first pestling and then pressing a pellet of 350 mg of KBr (Sigma-Aldrich; FT-IR grade) and 5 mg of pretreated biomass. A blank was prepared with 2.4 mg of microcrystalline cellulose (Avicel PH-101, Fluka) in 350 mg KBr, corresponding to the amount of cellulose present in the samples. The spectra of the pretreated biomass samples were corrected with the spectrum of the blank to obtain the mere lignin signal. We highlight that the thus obtained spectra are in very good agreement with spectra of pure lignin, where the influence of autohydrolysis with 2-naphthol on the lignin structure[9, 10] was studied.

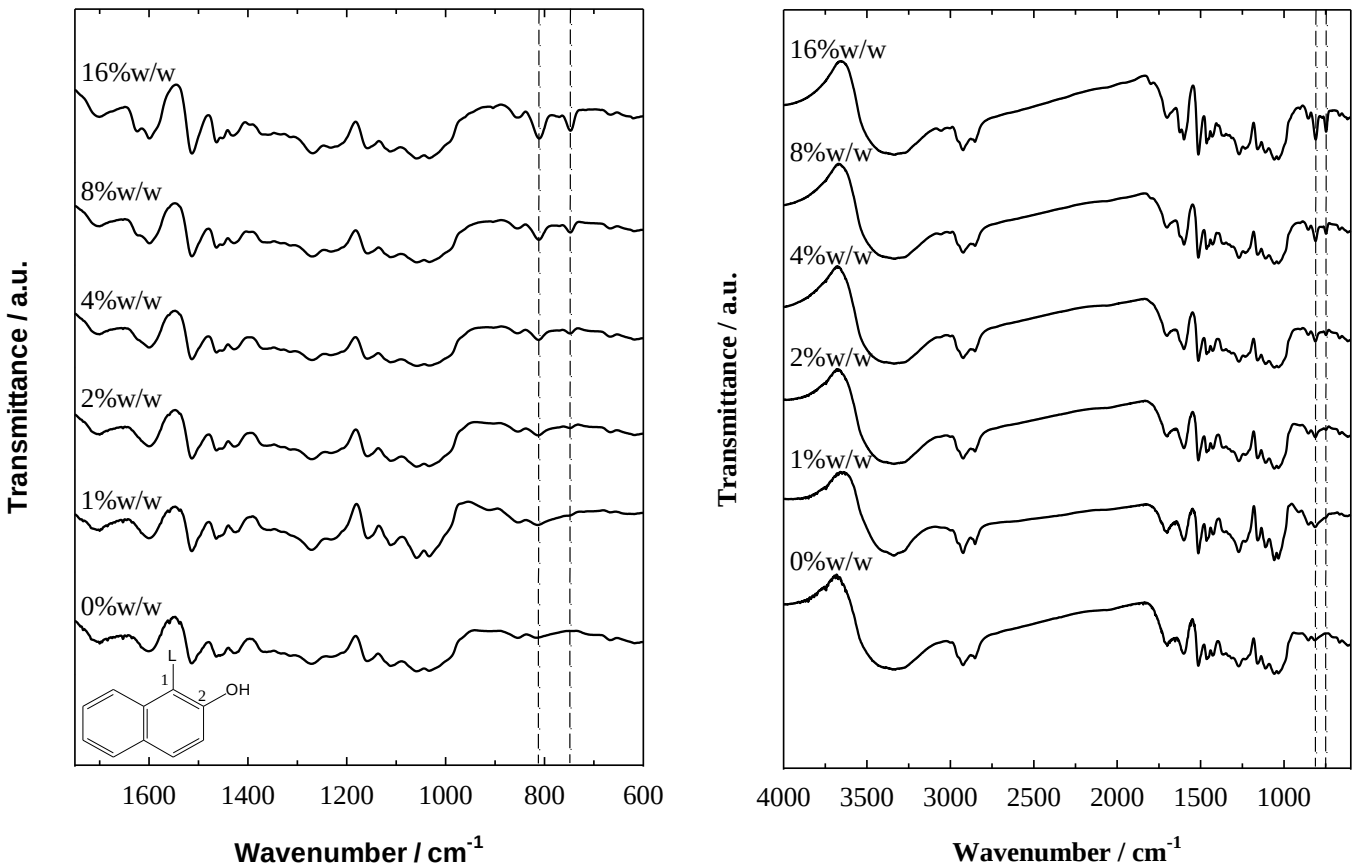


Fig. S11 FTIR spectra of lignins from spruce pretreated with different concentrations of 2-naphthol (0-16%w/w of biomass). Left: 1750-600nm; right: 4000-600nm; signals at 815 and 750 cm^{-1} are characteristic of 1,2-disubstituted naphtalenes[9, 11].

SEC analysis – Size-exclusion chromatography (SEC) analysis was carried out with lignin residues that had been pretreated without and with 16%w/w 2-naphthol (relative to biomass) and then isolated by enzymatic hydrolysis with an excess dose of enzymes (180 FPU/g cellulose, compare Fig. S14). To enhance lignin extractability, about 1.5 g of air-dried residues were ball milled using a rotary ball-mill (Retsch S100) with eight zirconium oxide balls (Ø10 mm) at a rotation speed of 250 min^{-1} for 20 h. 350 mg of the ball milled residues were then extracted with 40 ml of a 2:1 (v/v) dimethyl sulfoxide (Fluka, 99.8%):1-methylimidazole (Sigma-Aldrich, 99%) mixture[12]. The extraction was carried out in the batch reactor for 24 h at 80°C and a stirring speed of 400 min^{-1}. This procedure allowed for high lignin extraction yields of 66.8% (control) and 91.7% (16%w/w 2-naphthol), thereby enhancing the informative value of the samples. The extracts were further diluted with the solvent mixture to a lignin concentration of 2 g/L and then analysed by SEC as described elsewhere[7] without further sample preparation.

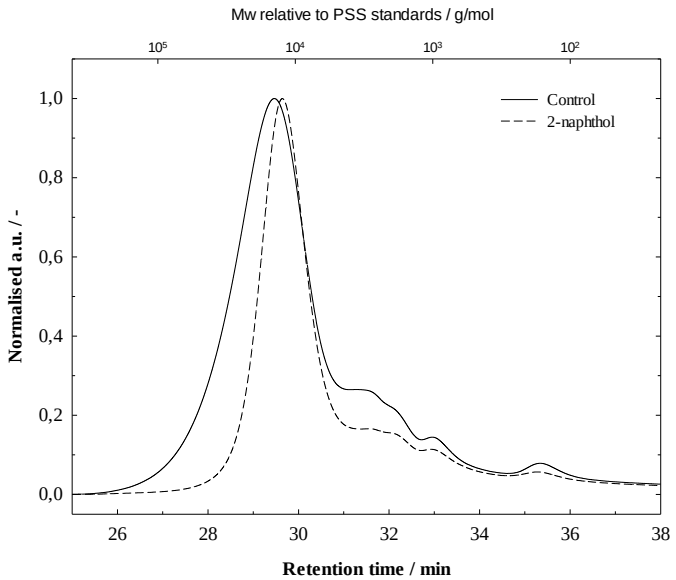
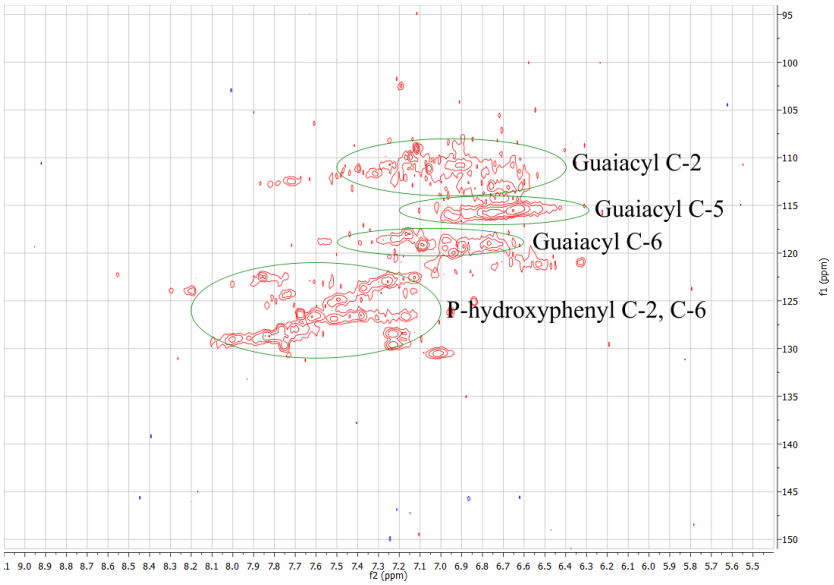
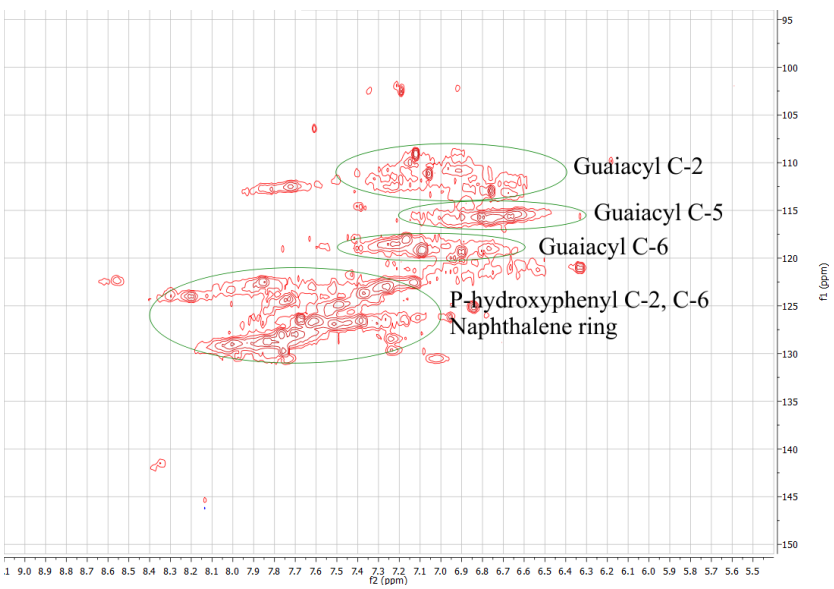


Fig. S12 Size-exclusion chromatography characterisation of lignin isolated from spruce pretreated without (control) and with 2-naphthol (16%w/w of biomass).

NMR spectroscopy – $^{13}C–^{1}H$ heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) measurements were carried out with lignins that had been isolated from spruce pretreated without and with 16%w/w 2-naphthol (relative to biomass). The isolation procedure involved enzymatic hydrolysis and ball milling as described previously for SEC analysis. Samples were then prepared by dissolving 28 mg of lignin residues in 0.75 ml of DMSO-d6 (Armar chemicals, 99.8% atom% D). NMR spectra were recorded at ambient temperature on a Bruker Avance III HD 500 MHz spectrometer using a standard Bruker pulse program[10, 13], allowing for a quantitative estimation particularly within the aromatic signals. The number of β-O-4 structures as well as the proportions of unsubstituted C-2, C-5 and C-6 carbon atoms in the guaiacyl rings were quantified based on measuring the volume integrals in the 2D HSQC spectra[10, 13].



a)



b)

Fig. S13 Aromatic region of the $^{13}C–^{1}H$ HSQC spectra of lignins isolated from (a) spruce pretreated without and (b) with 16%w/w 2-naphthol (relative to biomass).

Table S2 Structural data quantified by NMR for lignins isolated from pretreated spruce

Lignin Sample	β-O-4	Guaiacyl C-5/C-2	Guaiacyl C-6/C-2
Control	-	0.75	0.45
2-naphthol pretreated	-	0.71	0.79

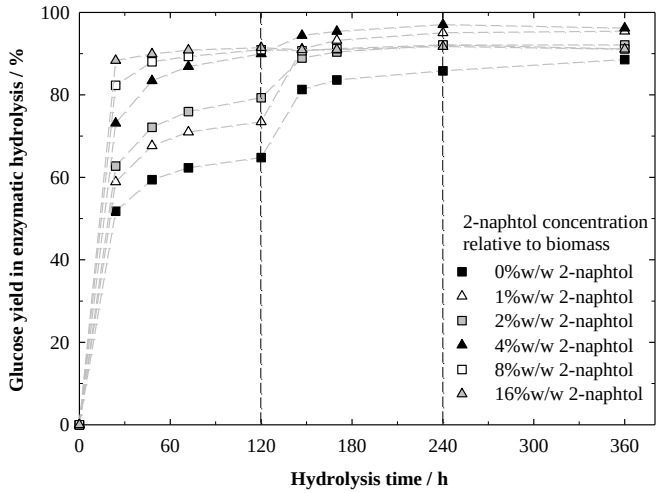
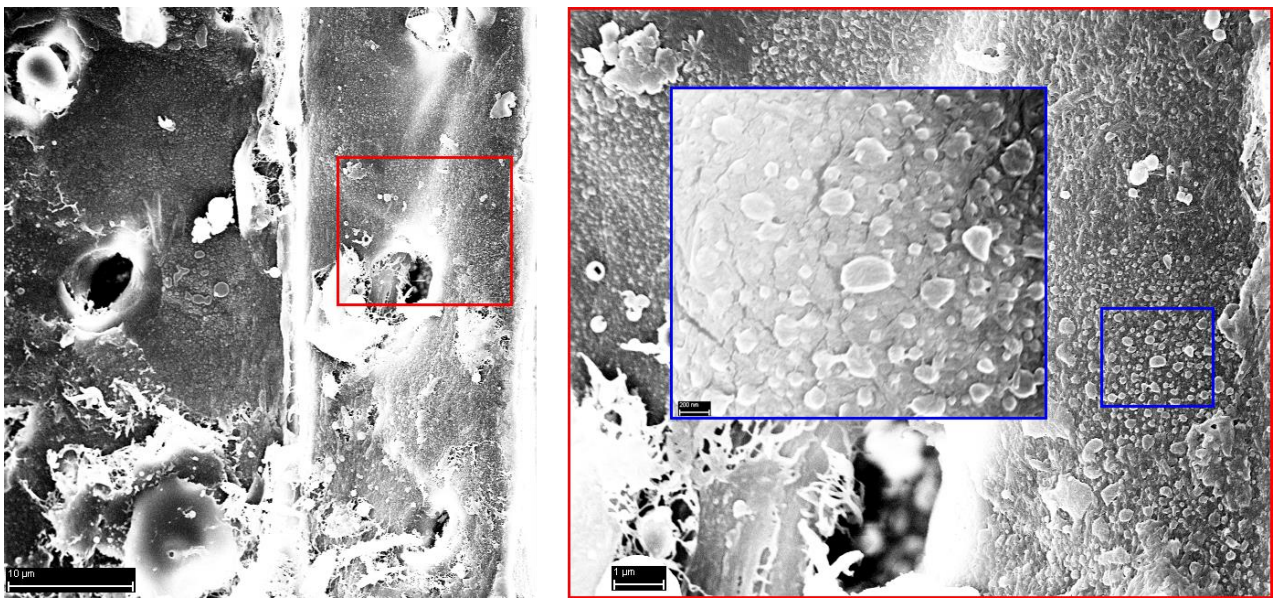


Fig. S14 Progress in enzymatic hydrolysis of spruce pretreated with different concentrations of 2-naphthol. Hydrolysis conditions: 1%w/w cellulose, initial enzyme dose 60 FPU/g cellulose. The same dose was added after 120 and 240 h (dashed vertical lines). Error bars not shown; all $\sigma < 2.6\%$.



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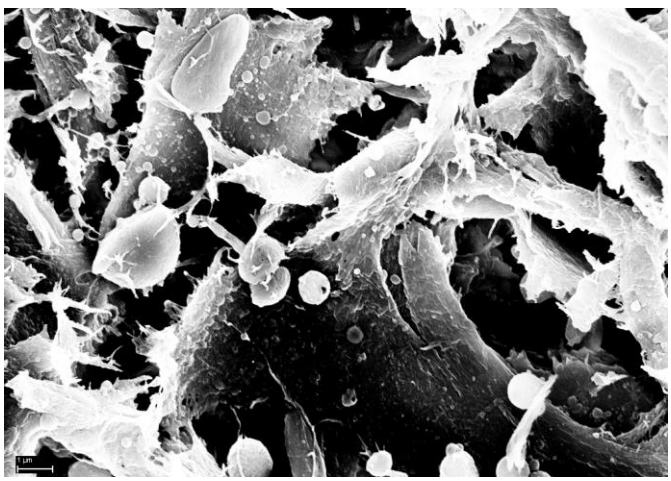

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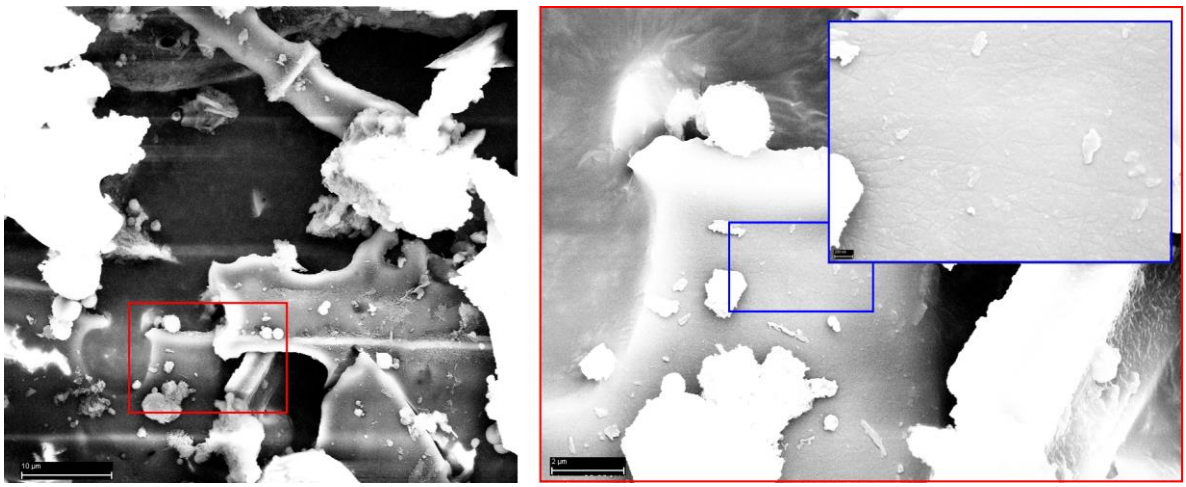

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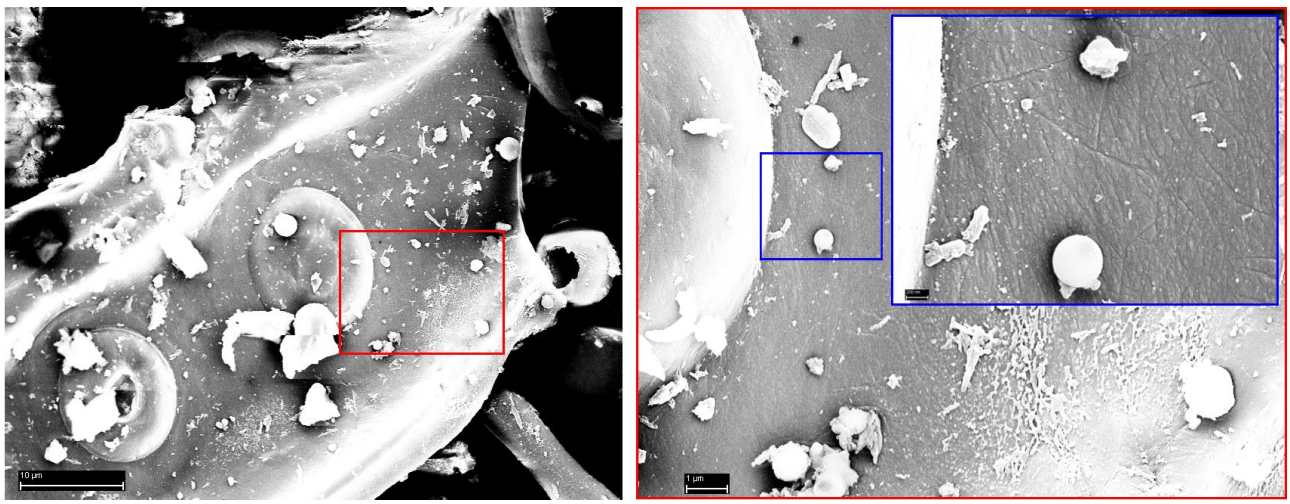

b)

Fig. S15 SEM images of lignin residues isolated from (a) spruce pretreated without and (b) with 16%w/w 2-naphthol (relative to biomass).

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