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Lignin repolymerisation in spruce autohydrolysis pretreatment increases cellulase deactivation†

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This study presents a modified autohydrolysis pretreatment which helps to overcome the recalcitrance of softwood for enzymatic hydrolysis of its cellulose. Autohydrolysis pretreatments of spruce wood were performed with 2-naphthol, which prevents lignin repolymerisation reactions, thereby increasing the enzymatic digestibility of cellulose by up to 64%. The negative influence of repolymerised lignin structures on enzymatic hydrolysis was confirmed by the addition of resorcinol in autohydrolysis, which is known to promote repolymerisation reactions and decreased the biomass digestibility. Several analyses were performed to study the underlying mechanism of this effect on hydrolysis, indicating that cellulolytic enzymes are adsorbed and deactivated especially by repolymerised lignin structures, which accounts for the high differences in biomass digestibility. It was shown that lignin repolymerisation significantly increases its specific surface area through modification of the lignin nanostructure, which is supposed to increase the unproductive binding of enzymes.

1. Introduction

Lignocellulosic biomass like wood, agricultural side products or energy crops is a potential renewable source for the production of chemicals and fuels. An intensively studied approach is the biochemical upgrading route by enzymatic hydrolysis of cellulose and hemicellulose to monomeric sugars, followed by fermentation to the final product. The lignin fraction of the biomass can be burned for the production of process heat and power but is also a prospective source for the production of aromatic chemicals due to the abundance of aromatic structures in the lignin polymer.

The lignin and its entanglement with cellulose and hemicellulose however, protects the biomass from external influences like enzymatic attacks. Lignin can hinder the enzymatic hydrolysis by acting as a physical barrier, restricting the accessibility of cellulose to enzymes,[2] but also by non-productive binding of cellulolytic enzymes.[2–4] The hindrance by lignin is one of the main reasons why a pretreatment capable of breaking down the lignocellulosic structure, or even removing the lignin, is necessary prior to the enzymatic hydrolysis of the biomass.

Autohydrolysis pretreatment methods like hot-water and steam pretreatment are attractive regarding their cost-savings potential.[5] They do not require acid, base or solvent chemicals and accordingly simplify a biorefinery process. The need for neutralisation chemicals is reduced[6] and the removal of a lignin solvent, which can be inhibitory to enzymes and fermentative microorganisms,[5] is not necessary. In particular steam pretreatments are of commercial relevance, as they allow for high biomass loadings and can help defibrating[7] the biomass by the rapid release of pressure (steam explosion). In fact, steam explosion is one of only a few cost-effective pretreatment technologies that have been advanced to pilot scale and commercialised application.[8]

Softwood is an interesting feedstock, being a fast and straight growing tree and the dominant lignocellulosic feedstock available in the Northern hemisphere, therefore viewed as a potential source for fermentable carbohydrate in the United States, Canada and Scandinavia.[7] However, hot-water and steam pretreatments on softwood are not effective for subsequent enzymatic hydrolysis.[9] Softwood like spruce is known to be especially recalcitrant to enzymatic hydrolysis, having even been called a "worst-case scenario" as a feedstock.[10] The high lignin content[7] and its great degree of cross-linking[11] are a major obstacle for the disintegration of the wood structure. Next to that, lignin that had been isolated from pretreated softwood was observed to exhibit stronger enzyme-lignin interactions and inhibitory effects than lignin from other sources.[12]

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To the extent of our knowledge, only two pretreatment methods allow for high cellulose conversions from softwood with moderate enzyme dosages. The addition of an acid catalyst like sulfur dioxide (SO_2)[9,13] and organosolv pretreatments[9,14] which remove lignin from the biomass. The complexity of those processes however is a challenge and operations that remove lignin are seen as too expensive for the bioconversion of lignocellulose into fuels.[11] While organosolv pretreatments lead to an outstanding digestibility due to the almost complete lignin removal from softwood, processes involving SO_2 just partly remove lignin as lignosulphonates but sulphonate the remaining lignin, increasing its hydrophilicity.[18,19] This is supposed to be beneficial for enzymatic hydrolysis by reducing hydrophobic lignin-enzyme interactions[18–20] and reflects the importance of lignin conditioning in softwood pretreatment.

In autohydrolysis pretreatments, hemicellulose is easily solubilised and removed, while lignin is practically not removed and remains in the solid phase together with cellulose.[5] This is one reason why autohydrolysis pretreatments are not effective for softwood whose lignin forms a particularly big obstacle for enzymatic hydrolysis. Another important factor might be repolymerisation reactions (sometimes also referred to as condensation reactions) of the lignin which take place during the pretreatment. In autohydrolysis, organic acids like acetic acid are released from hemicellulose[6] leading to moderate acidic conditions of typically pH 2–4, depending on the type of biomass, load and pretreatment severity. Researchers have found that under acidic treatments of wood, carbonium ions are formed in the lignin molecule, that are responsible for the repolymerisation reactions.[17,21,22] In today's chemical terminology, those three-coordinate carbon ions are denoted as carbenium ions,[23] but will be referred to as carbocations here to avoid misconceptions with existing literature. Those carbocations have been identified as intermediates in lignin depolymerisation reactions, especially in the cleavage of β-aryl-ether linkages as shown in Fig. 1 (route a–c). On the other hand, the electrophilic carbocations are also supposed to form, through substitution, stable C–C bonds with the electron rich carbon atoms of the aromatic rings present in lignin[17,21,22] (Fig. 1 route a and d). High-molecular weight and repolymerised lignin structures are formed.[21,24,25] It has further been reported that also the phenolic extractives of wood may react in that way with lignin.[25] We suspect that such repolymerised lignin structures form an additional obstacle for the enzymatic hydrolysis of cellulose.

Guaiacyl units in lignin can undergo repolymerisation reactions in autohydrolysis more easily[26] and it has been suggested that – even in the presence of other monolignols – these reactions preferably take place among the guaiacyl units themselves.[27] It is interesting to note that different studies[28,29] reported how a decrease in the Syringyl(S)/Guaiacyl(G) ratio of lignin in biomass also gave rise to a significant decrease in glucose release by enzymatic hydrolysis after an autohydrolysis pretreatment. Remarkably, the S/G ratio had no influence on sugar release when no pretreatment was carried out. Next to the more difficult cleavage of bonds in samples with low S/G ratios[28] (less β-O-4 and more 5–5 and β-5 linkages[30]), an increase in repolymerisation reactions during pretreatment with increasing guaiacyl content might play a role in this effect.

The S/G ratio in softwoods is exceptionally low,[30] suggesting that repolymerisation reactions play an important role in autohydrolysis. A method for suppressing undesired lignin repolymerisation reactions is the use of carbocation scavengers. Wayman and Lora[21] first reported that certain aromatic

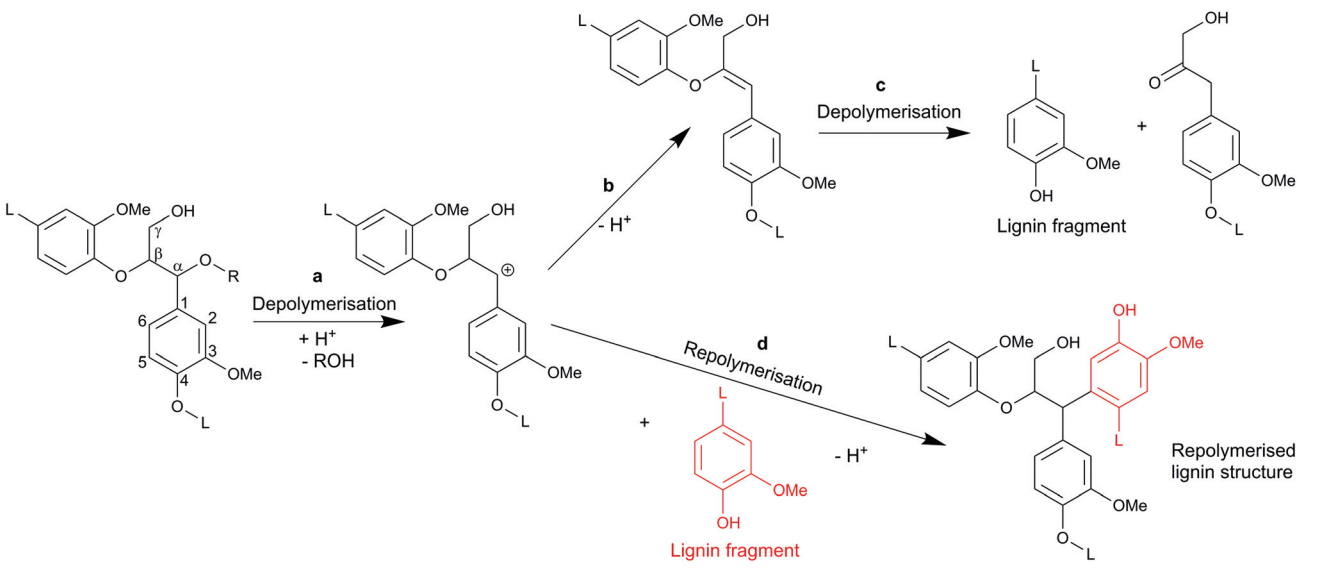

Fig. 1 Scheme for reactions of lignin in acidic media adapted from Voitl *et al.*[15] (a) cleavage of β-O-4 bond and formation of carbocation;[16] (b) elimination of β-proton giving rise to an enol ether;[16] (c) acid hydrolysis of enol ether;[16] (d) repolymerisation reaction.[17]

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compounds can act as scavengers in the autohydrolysis of aspen and increase the yield of organic solvent-extractable lignin, allowing to obtain a highly delignified pulp. The scavengers are believed to compete with the aromatic rings present in the lignin for the formed carbocations and prevent repolymerisation reactions. 2-naphthol was found to be a very effective additive for preventing repolymerisation, yielding a lignin of lower molecular weight[1,22] with greatly enhanced extractability.[21,22,24,31]

We have recently shown that the use of carbocation scavengers in the autohydrolysis of lignocellulosic biomass can also improve its enzymatic hydrolysability.[32] The cellulose conversion in the enzymatic hydrolysis of spruce was improved up to 46% by adding 2%w/w (relative to biomass load) of 2-naphthol to the hot-water pretreatment. Surprisingly, the enhanced digestibility was not based on an increased lignin removal during the pretreatment. The effect was therefore attributed to a potentially different lignin structure in the pretreated biomass.

The present manuscript addresses two questions of an autohydrolysis pretreatment with carbocation scavengers. First, what is its potential for overcoming the high recalcitrance of softwood, *i.e.* to what extent can sugar yields be enhanced? Second, in which way do lignin structures that repolymerise in autohydrolysis hinder the enzymatic hydrolysability?

2. Experimental

2.1. Enhancing softwood digestibility

Biomass. Spruce cut in spring 2012 in Villigen (canton of Aargau, Switzerland) was debarked, split with an axe, chipped and knife milled (SM200 cutting mill; Retsch) through a 1 mm screen size. All material was sieved to less than 1 mm and greater than 0.18 mm (AS200 vibratory sieve shaker; Retsch). Particles smaller than 0.18 mm were discarded. The dry matter (93.35 ± 0.18%) and the composition were determined by standard National Renewable Energy Laboratory (NREL) methods:[33,34] Glucan 44.49 ± 0.51%, Mannan 18.33 ± 0.12%, acid soluble lignin (ASL) 5.00 ± 0.08%, acid insoluble lignin (AIL) 26.79 ± 0.17%, extractives 5.13% and ash 0.28% ± 0.01 (total 100.02%).

Pretreatment. Pretreatment experiments were carried out in a batch screening reactor (MRS 5000; Parr Instruments) which consisted of six 75 ml titanium lined reactors equipped with pressure sensor, thermocouple, a suspended magnetic stirrer and an external electrical heating (modified to provide 300 W for each reactor). The reactor was loaded with 2.5 g of undried spruce and 39.2 g of water. Optionally, 118.5 mg of 2-naphthol (Sigma-Aldrich, 98%) or 45.2 mg of resorcinol (Chemie Brunschwig, 98%) were added, corresponding to 4.74 or 1.81% w/w of biomass, respectively, or 0.205 and 0.103 mol/mol lignin C_9 unit. The reactor was purged three times with nitrogen (10 bar) prior to heating and the stirring speed was set to 400 min^{-1}. The (crucial) heating interval from 100 °C to the reaction temperature of 210 °C took about 12 min and then the pretreatment lasted for varying times up to 4 h (compare Table S1†). Submerging the reactor in a water bath at the end of the pretreatment allowed for fast cooling below 100 °C in less than one minute. The pretreatment severity R_0 was calculated as defined by Overend *et al.*[35] also taking the heat-up time of the reactor into account (details see ESI†). The contents of the reactor were vacuum filtered (Filter paper Macherey-Nagel Mn615), recording volume and pH (Hamilton Polilyte HT 120 electrode) of the filtrate. The solids were washed with 300 ml of boiling water, then weight and moisture of the recovered biomass was recorded for mass balance calculations.

Compositional analysis of biomass and liquor from pretreatment. The content of glucan, mannan, acid-soluble lignin (ASL) and acid-insoluble lignin (AIL) in the pretreated biomass was determined by a modified standard NREL procedure[33] downscaled by a factor of 56. Analysis of sugars and byproducts in the pretreatment liquor was done as specified by Sluiter,[36] similarly downscaled as the biomass compositional analysis. All biomass and pretreatment liquor analyses were done in triplicate and duplicate, respectively, and single standard deviations are reported with the mean in this work. The complete experimental procedure is provided in the ESI.†

Enzymatic hydrolysis. Enzymatic hydrolysis of the biomass was conducted according the NREL standard procedure using 1%w/w cellulose loading.[37] The following changes were made: sodium azide at a final concentration of 0.01 g L^{-1} was used instead of antibiotics and the pH was adjusted to 5.0 (0.05 mol l^{-1} sodium citrate buffer after sample preparation). Accelerase 1500 (Genencor; lot number 4901298419), with an activity of 26 filter paper units (FPU) ml^{-1},[38] was used with a final concentration of 60 FPU g^{-1} cellulose in the sample preparation. The samples were incubated in a shaker (Multitron; Infors-HT) with a shaking throw of 50 mm at 50 °C and 210 rpm for 120 h and then analysed for sugars in the supernatant. All hydrolysis experiments were carried out in triplicate and single standard deviations are reported with the mean.

Sugar analysis. Sugar analysis by HPLC was performed using a Waters 2695 Separation Module equipped with a Waters 410 Differential Refractometer and a Bio-Rad Aminex HPX-87H column.

2.2. Effect of lignin repolymerisation on enzyme deactivation

Biomass. Sawdust from a roughly 30 year old, debarked spruce, cut in winter 2012 in Bueren a.A., Switzerland, was milled, sieved and analysed analogue the wood chips in section 2.1. The dry matter (74.63 ± 0.39%) composition was determined to: Glucan 45.18 ± 0.55%, Mannan 17.74 ± 0.28%, ASL 4.77 ± 0.42%, AIL 28.73 ± 0.06%, extractives 4.89% and ash 0.22% ± 0.01 (total 101.53%).

Pretreatment and biomass analysis. Pretreatment was carried out as described in section 2.1 but with varying the amounts of 2-naphthol between 1, 2, 4, 8 and 16%w/w of the biomass (compare Table S1†). Analysis of the pretreated biomass was carried out as described in section 2.1.

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Isolation of lignin residues. Enzymatic hydrolysis was carried out as described in section 2.1, with adding a second and third enzyme dose of 60 FPU g^{-1} of initial cellulose after 120 and 240 h. The lignin-rich residues from the enzymatic hydrolysis were then subjected to a protease treatment to remove solid-bound cellulases.[39,40] The solids from enzymatic hydrolysis were recovered and centrifugation-washed three times with 8 ml of a pH 2.5 HCl solution and finally with 5 ml of a pH 9.6 Na_2CO_3/$NaHCO_3$ buffer in a 10 ml centrifuge tube. Thereafter, 70 μl of a 100 mg ml^{-1} protease solution (Type XXIV, Sigma-Aldrich) and 7 ml of the pH 9.6 buffer were added to each tube. This corresponds to a lignin concentration of 50 mg ml^{-1} and a protease dosing of about 0.02 mg mg^{-1} lignin. The protease treatment was carried out for 48 h at 37 °C in a shaking water bath (Model 1086; GFL). After the treatment, the samples were centrifuged and the solids were washed again three times with 8 ml of pH 2.5 HCl and then freeze dried for 36 h. The protein content of the lignin residues before and after the protease treatment was evaluated indirectly by nitrogen analysis (CHN-900 elemental analyser; Leco).

Hydrolysis of pure cellulose in the presence of lignin residues. Hydrolysis of pure microcrystalline cellulose (Avicel PH-101) in the presence of the isolated lignin-rich residues was performed to study the influence of lignin on enzyme deactivation.[12,39] Hydrolysis was prepared in a similar manner as described in section 2.1. Samples of 5 ml were prepared with a cellulose concentration of 1%w/w and a lignin concentration of 1%w/w. The enzyme dose was 10 FPU g^{-1} cellulose. The hydrolysis slurry was sampled 4, 24, 48 and 120 h after the start of the hydrolysis.

BSA treatment. A separate set of enzymatic hydrolyses was prepared with treating the pretreated biomass with bovine serum albumin (BSA) before adding the celullases. Hydrolysis was prepared in a similar manner as described in section 2.1. 5 ml samples were prepared with biomass as to obtain a cellulose concentration of 1%w/w in each vial. 0.5 ml of a 0.05 mg ml^{-1} BSA solution (≥98%, heat shock fractionated, Sigma-Aldrich) were added to each vial, corresponding to a final BSA concentration in the sample of 5 g l^{-1} as employed by Brethauer *et al.*[41] and about 0.5 g BSA g^{-1} lignin. The samples were placed for 24 h in the incubator at 50 °C before adding the cellulases (60 FPU g^{-1} cellulose) and the enzymatic hydrolysis was allowed to proceed as described in section 2.1.

SEM analysis of lignin residues. The surface of the isolated lignin residues was characterized by scanning electron microscopy (SEM). Samples were coated with a platinum layer of 3 nm in a sputter coater (MED 010, Bal-Tec) and analysed with a Gemini1530 (Zeiss) electron microscope operated at an acceleration voltage of 2 kV.

BET and BJH analysis of lignin residues. The specific surface area and pore volume distribution of the isolated lignin residues were analysed by the Brunauer–Emmett–Teller (BET) and the Barret–Joyner–Halenda (BJH) method, respectively. The analyses were carried out on a Micromeritics TriStar 3000 gas adsorption device using at least 0.1 g of substrate and nitrogen as adsorbate. The BET analysis was carried out in duplicate and single standard deviations are reported with the mean in this work. A lignin sample of non-pretreated biomass, isolated by ball milling and enzymatic hydrolysis, was analysed as well. 3 g of spruce 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. Enzymatic hydrolysis of the milled biomass was prepared as described in section 2.1 with an excess enzyme dose of 180 FPU g^{-1} cellulose, allowing for a cellulose conversion of 90%. Samples were then freeze dried for 36 h before analysis.

3. Results and discussion

3.1. Enhancing softwood digestibility

Pretreatment with a crossing and blocking agent for lignin fragments. Autohydrolysis pretreatments were carried out in the presence of 2-naphthol, resorcinol and without additive (control). 2-naphthol is argued to be an effective lignin carbocation scavenger for two reasons. It is a strong nucleophile so it can attack the positively charged carbocations plus it preferably undergoes just a single electrophilic substitution, thereby not promoting lignin crossing reactions.[21] 2-naphthol is easily substituted in position 1,[42] but not in position 3 as then the transition state can only be stabilised by the OH group in a non aromatic structure[21] (see Fig. 2).

In contrary to 2-naphthol, the addition of resorcinol at low concentrations was found to promote the formation of repolymerised lignin and increase its molecular weight.[1,21] Resorcinol is a strong nucleophile with its aromatic ring being activated by two OH groups which are *ortho* and para directing towards electrophilic substitution. The resulting three activated positions allow for several substitutions to occur (up to three[43]) and increase the cross-linking of lignin fragments, as shown in Fig. 3.

Pretreatment severities between $\log R_0$ = 4.5 and 5.6 as defined in (ESI eqn (4)†) were tested in the experiments with the additives. A severity of 5.1 was enough so as to release all acids from the hemicellulose and decreased the pH in the liquor to around pH 3. Increasing severity did not decrease pH any further (Fig. S1†). Hemicellulose (represented as mannan)

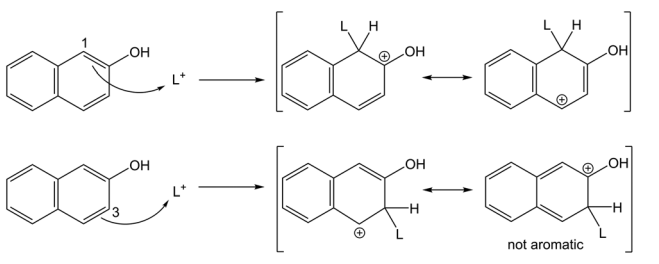

Fig. 2 Reaction scheme showing the nucleophilic attack of 2-naphthol on a lignin carbocation (L+) with the substitution occurring in C-1 (top) and C-3 position (bottom). Not all resonance structures shown.

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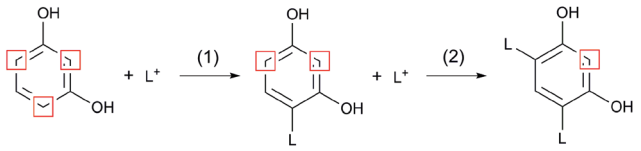

Fig. 3 Reaction scheme adapted from Lora and Wayman[1] showing the first (1) and second (2) reaction of resorcinol with a lignin carbocation (L+). Positions most reactive towards electrophilic aromatic substitution are highlighted.

content in all pretreated biomasses was low (Fig. S2a†) as it was easily dissolved, around 76% are already removed at a severity of 4.5. Cellulose content gradually decreased with increasing severity as more cellulose got hydrolysed and dissolved (Fig. S2a†). The AIL content rises accordingly, but no influence of the additives on the removal of lignin from the biomass is noticeable (Fig. S2b†). ASL just makes up a minor fraction of the lignin. Its content was increased with 2-naphthol and resorcinol (Fig. S2b†), probably due to their aromatic molecules being integrated into the lignin structure, thereby increasing UV-absorption in the ASL measurement. Besides, the suppression of lignin repolymerisation with 2-naphthol might also increase its solubility and thereby ASL content.

Sugar yields. The glucose yields in the enzymatic hydrolysis of the pretreated biomasses are shown in Fig. 4. Untreated spruce had a cellulose digestibility of 5.6%. The digestibility of the control increased with severity as a result of the more intensive pretreatment. The maximum glucose release was 63% for a severity of 5.4 and decreased again with higher severity. The latter can be explained with the increasing lignin content in the biomass (Fig. S2b†), as lignin can adsorb and deactivate enzymes in hydrolysis. Repolymerisation reactions of lignin, that should be more pronounced at higher severities, can play a role as well. The prevention of repolymerisation reactions with 2-naphthol increased the cellulose digestibility in every experiment. The digestibility could be enhanced by up to 64% compared to the control at a severity of 5.1. Remarkable for softwood, an almost complete conversion of cellulose was possible. As a proof of concept, increasing the lignin repolymerisation by adding resorcinol to the pretreatment decreased the biomass digestibility in every experiment and as far as 26% at a severity of 5.4. Regarding the lower impact of resorcinol in contrast to 2-naphthol, it should be considered that it was also added in a lower concentration (0.103 compared to 0.205 mol/mol lignin C_9 unit).

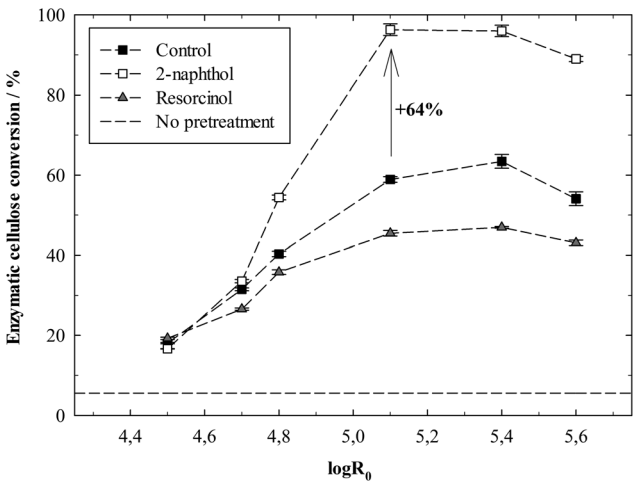

Fig. 4 Cellulose conversion/glucose yields in the enzymatic hydrolysis of spruce after autohydrolysis pretreatments at different severities with 2-naphthol (4.7%w/w of biomass), resorcinol (1.8%w/w of biomass) and no additive (control). Yields do not account for cellulose degradation during pretreatment. Hydrolysis conditions: 1%w/w cellulose, 60 FPU g^{-1} cellulose.

Fig. S3† shows the yields of mannose and glucose being dissolved in the pretreatment liquor from the biomass. The pretreatment additives had no influence on the sugar yields in the pretreatment liquor. It can thus be assumed they did not affect sugar dissolution and degradation.

The highest total glucose yield, summing up the glucose yields from enzymatic hydrolysis and pretreatment liquor, was 81% and reached at a severity of 5.1 with 2-naphthol (Fig. S4a†). The gradual degradation of cellulose in the pretreatment (compare Fig. S2a†) reduced the yields, especially at very high severities. Next to that, in particular the fast degradation of mannose reduced the total sugar yields, which sum up all yields from cellulose and hemicellulose (Fig. S4b†). The highest total sugar yield of 58% was likewise reached at a severity of 5.1 with 2-naphthol.

Characteristics of a scavenger process. The results point out the general challenge that autohydrolysis pretreatments have to deal with: hemicellulose gets dissolved and degraded fast, while cellulose, especially in softwood, needs a harsh pretreatment to become digestible. A common approach to decrease the fast degradation of hemicellulosic sugars from softwood is a two-stage pretreatment process.[44] A first stage under mild conditions dissolves and recovers hemicellulosic sugars, followed by a second stage under harsher conditions in order to increase the cellulose digestibility. In such a process, the carbocation scavenger could be added in the harsh pretreatment with a high severity – where it has a particularly high effect (compare Fig. 4) – and further enhance the digestibility. This should allow for high overall sugar yields from softwood. Useful for comparison, a review on one and two-stage acid softwood pretreatment methods by Galbe and Zacchi[44] presented the highest total sugar yield of 82% for an optimised two-stage dilute-sulphuric acid pretreatment.[45] A mix of softwood was pretreated and then enzymatically hydrolysed under conditions equal to this work (60 FPU g^{-1} cellulose).

Considering the fact that the not-yet optimized pretreatment with scavengers allowed for total glucose yields of 81%, an additional recovery of the hemicellulose fraction should allow for comparable or even higher sugar yields in a simple autohydrolysis process. Moreover, preliminary experiments show that 2-naphthol is even more effective in (dilute) acid

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pretreatments, as carbocations are especially formed under acidic conditions[17] and considerable lignin repolymerisation can be expected. Further preliminary experiments show that 2-naphthol is also effectively applicable to a pure steam pretreatment by simple mixing of the biomass with the 2-naphthol powder before the pretreatment.

Pretreatment severities that allowed for nearly complete cellulose conversions ($\log R_0 > 4.8$) resulted in 2-naphthol conversions of 95% and higher (Fig. S5†). This corresponds to a remaining 2-naphthol concentration of approximately 0.5%w/w of the cellulose in the pretreated biomass. Considering the further processing after pretreatment, it has to be noted that the activity of cellulases can be inhibited by monomeric[46] or oligomeric[47] phenolic compounds like 2-naphthol. Enzymatic hydrolysis experiments of Avicel (microcrystalline cellulose) in the presence of 2-naphthol did however not reveal an inhibition of enzymes, even at very high 2-naphthol concentrations of 35%w/w of cellulose (Fig. S6†). Next to that, phenolic compounds have also been suggested to exert an inhibitory effect in fermentation.[48] The fermentation of glucose in the presence of 2-naphthol revealed that the growth of yeast organisms can indeed be inhibited by 2-naphthol, which was estimated by absorption measurements of the fermentation broth (Fig. S7a†) that correlate with the concentration of yeast cells in the liquid.[49] 2-naphthol concentrations below 1%w/w of glucose however allowed for complete glucose conversions (Fig. S7b†) and did neither influence ethanol yields in a 24 h fermentation (Fig. S7c†). It can thus be assumed that a process with 2-naphthol conversions of more than 95% as presented here should not be afflicted by noticeable yeast inhibition.

Next to increasing the biomass digestibility, the prevention of lignin repolymerisation could at the same time add value to the obtained lignin fraction. Such a lignin, with a more uniform, lower molecular weight and introduced functionalities by the scavenger like aryl groups could *e.g.* be interesting for the use as a blend in polymers.[31] The production of aromatic monomers from lignin could be enhanced as well due to less aromatic C–C bonds that are difficult to cleave.

3.2. Effect of lignin repolymerisation on enzyme deactivation

As presented in the previous section, the suppression or increase of lignin repolymerisation did not influence lignin removal from the biomass in autohydrolysis. Still, its enzymatic digestibility was considerably affected. Two possible mechanisms were considered being responsible for this effect. On the one hand, repolymerised lignin structures may "enwrap" or partly cover the cellulose fibres and thereby block the access of enzymes. On the other, it is known that enzymes can be deactivated by irreversible binding[2] and possibly even denaturing[39,50] on lignin. Those processes could occur more pronounced on repolymerised lignin structures.

In order to study both possible mechanisms, biomass samples with a varying degree of repolymerised lignin were prepared and underwent several experimental procedures, as outlined in Fig. 5. Autohydrolysis treatments of spruce were carried out with varying 2-naphthol concentrations between 0 and 16%w/w of the biomass (compare Table S1†) at a high severity of $\log R_0 = 5.4$, which had allowed for a considerable effect of the scavenger on biomass digestibility (see Fig. 4).

The 2-naphthol concentration in autohydrolysis had neither a noticeable influence on pH (Fig. S8†) nor on the formation of dissolved byproducts and sugar degradation products detectable by HPLC (the main constituents acetic acid, HMF and furfural were quantified; Fig. S9†). Hence, those products were most likely not involved in reactions with the scavenger or lignin repolymerisation.

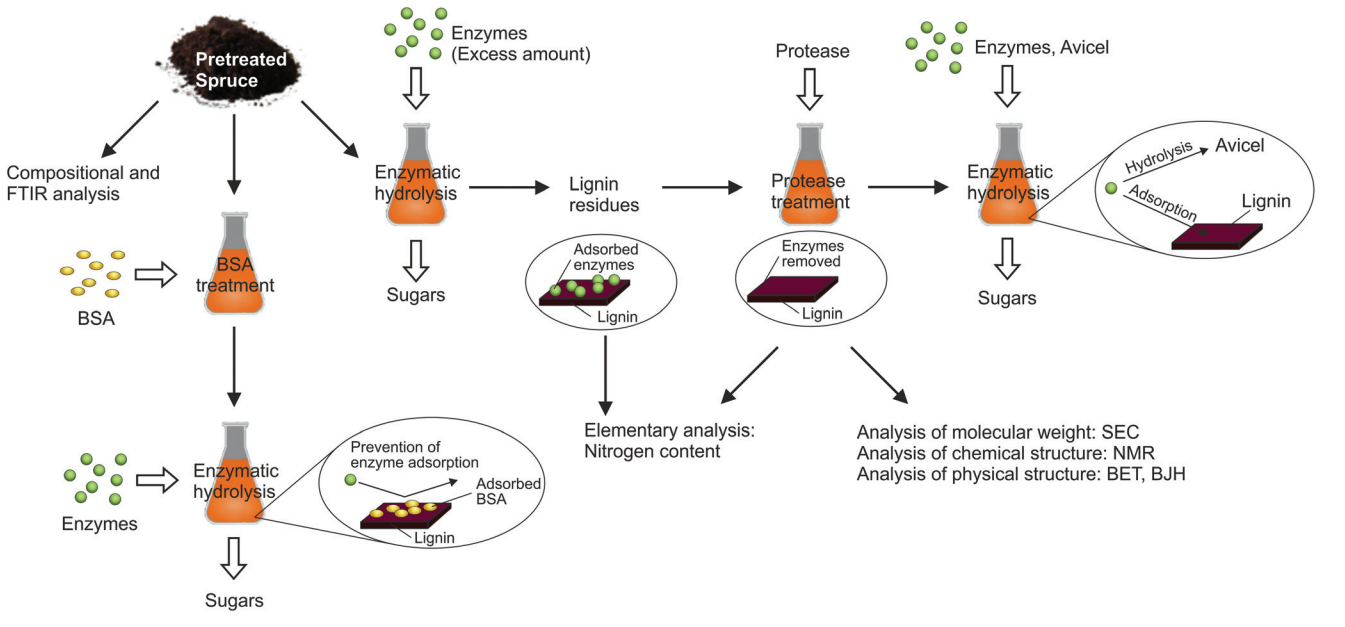

Fig. 5 Overview of experimental procedures for studying cellulase deactivation on spruce pretreated with different 2-naphthol concentrations.

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Effect of 2-naphthol on lignin structure. The data from the compositional analysis of the pretreated biomass (Fig. S10†) is consistent with the previous findings, showing that 2-naphthol did hardly influence lignin removal. The ASL content was however increased with increasing 2-naphthol concentration. As stated in section 3.1, the increasing ASL content may be attributed to more 2-naphthol being integrated into the lignin structure increasing its UV-absorption and/or to a higher solubility of the less repolymerised lignin.

The increased integration of 2-naphthol into the lignin structure with increasing concentration in autohydrolysis could be confirmed by FTIR analysis of the pretreated biomass. With increasing 2-naphthol concentration, a strengthening of IR signals at 750 and 815 cm^{-1} can be observed (Fig. S11†). This is characteristic for 1,2-disubstituted naphthalenes[51,52] like 2-naphthol substituted at position C-1 (as illustrated in Fig. S11†). The finding also shows that 2-naphthol preferably undergoes a single electrophilic substitution on position C-1 and acts as a blocking agent for lignin fragments in that way, since otherwise the strengthening of further signals (*e.g.* from 1,2,3-trisubstituted naphthalenes at 790 cm^{-1} (ref. 51)) should be observable. In the whole scanning range of 4000–600 cm^{-1}, no further significant signal changes could be observed (Fig. S11†), also indicating that 2-naphthol did not influence functional groups of the lignin detectable by FTIR.

The size-exclusion chromatography (SEC) analysis of lignin that had been isolated from the pretreated biomass gives information about changes in its molecular weight distribution (isolation and chromatography procedure provided in the ESI†). The use of 2-naphthol distinctly decreased the molecular weight and also lead to a narrower molecular weight distribution, as shown exemplarily in Fig. S12.† This indicates it was effectively preventing lignin repolymerisation, which generally leads to an increased molecular weight and a more heterogeneous lignin structure.[22] In addition, the lignin isolation procedure revealed that the organic solvent extractability of the lignin was enhanced by the use 2-naphthol, a characteristic that has been attributed to its less repolymerised structure.[21,22,24] The prevention of lignin repolymerisation by 2-naphthol was further disclosed by ^{13}C–^{1}H heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) measurements of the isolated lignins (isolation and NMR procedure provided in the ESI†). The quantity 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 can be quantified based on the HSQC spectra[31,53] and are shown in Table S2.† The use of 2-naphthol (16%w/w of biomass) did practically not affect the C-5/C-2 signal ratio, while the C-6/C-5 signal ratio was increased by 76% compared to the control, indicating a different substitution pattern of the aromatic nuclei. Under acidic conditions, repolymerisation reactions can occur predominantly between the C-6 site and the carbocation at the C-α position of adjacent lignin structures in softwood lignin[54,55] (compare Fig. 1), probably accounting for the reduced C-6 signal of the control. No signals from β-O-4 structures could be observed in the pretreated samples, likely meaning those bonds had completely been cleaved. Next to that, the NMR spectra can again show the incorporation of 2-naphthol into the lignin structure[31] (Fig. S13†).

Does repolymerised lignin decrease cellulose accessibility? In order to isolate possible "enzyme blocking" effects from "enzyme deactivation" effects of the lignin, the biomass underwent a BSA treatment before adding the cellulases for hydrolysis. The hydrophobic sites of BSA adsorb readily on hydrophobic surfaces[56,57] such as lignin. BSA is believed to adsorb selectively[58] on lignin, thereby disabling the unproductive binding of cellulases on it, which can enhance sugar yields[41,56] in enzymatic hydrolysis.

Without BSA treatment, increasing 2-naphthol concentration in the pretreatment up to 4%w/w steadily increased sugar yields in hydrolysis (Fig. 6). 4%w/w of 2-naphthol were sufficient to reach yields of 90% after 120 h of hydrolysis. Treating the biomass before hydrolysis with BSA however, allowed for a virtually complete cellulose conversion after 120 h of hydrolysis, independent of the 2-naphthol concentration. This reveals that the cellulose in all samples had been equally accessible. Lignin is not blocking digestible cellulose, preventing enzymes from accessing it, but repolymerised lignin seems to intensify the enzyme deactivation.

BSA treated samples, pretreated with low amounts of 2-naphthol (0–4%w/w) were digested slower, as can be seen from the hydrolysis conversions after 24 h. This indicates that, while certainly effective, the applied amounts of BSA could not occupy all binding sites on samples with more repolymerised lignin and more enzymes were deactivated.

Revealing the extent of enzyme deactivation by repolymerised lignin structures. In order to study the extent of enzyme deactivation caused by the mere lignins in the pretreated biomass, Avicel was enzymatically hydrolysed in their

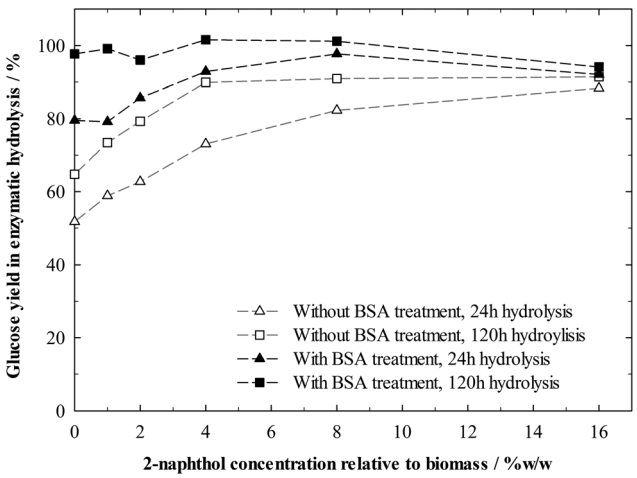

Fig. 6 Glucose yields in the enzymatic hydrolysis of spruce pretreated with different concentrations of 2-naphthol. One sample set underwent BSA treatment before enzymatic hydrolysis. Hydrolysis conditions: 1%w/w cellulose, 60 FPU g^{-1} cellulose, 120 h.

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presence. In that way, the cellulose substrate and the lignin are physically separated, allowing to visualise the absolute extent of enzyme deactivation by the lignins. Intrinsic influences of the lignocellulosic biomass on hydrolysis such as cellulose accessibility do not play a role.

Therefore, the lignins were first isolated by enzymatic hydrolysis from the pretreated biomass with an excess dose of enzymes. The progress of this hydrolysis over time with a successive increase of the enzyme dose is shown in Fig. S14.† Increasing 2-naphthol concentration in pretreatment continuously increased the speed of the cellulose digestion, suggesting that less enzymes were deactivated and the hydrolysis could proceed faster.

The biomass residues after hydrolysis consisted primarily of lignin as nearly all cellulose had been digested. However, enzymes from the hydrolysis were expected to be still adsorbed on the lignin, so the material was "cleaned" for the following Avicel hydrolysis by hydrolysing the adsorbed proteins with a protease treatment. The nitrogen content of the lignin residues before and after the protease treatment is shown in Fig. 7. This serves as an indirect indicator of the protein content, which can be increased by enzyme contamination.[3,39,59] The nitrogen content was significantly decreased by the protease treatment, showing that basically proteins, which can effectively be removed by proteases, account for the nitrogen content. The amount of proteins bound to the residues before protease treatment decreased with increasing amount of 2-naphthol in the pretreatment. This proves that enzyme adsorption plays a major role in the deactivation process and the pretreatment with 2-naphthol decreases the ability of lignin to bind enzymes in hydrolysis.

It is interesting to note that the protease treatment did not reduce the nitrogen content of all samples to the same level. A possible explanation could be that the access of protease to the adsorbed enzymes was restricted by the structure of the more repolymerised lignins.

The isolated and "enzyme cleaned" lignin residues could then be added to the enzymatic hydrolysis of Avicel. Hydrolysis experiments were carried out with concentrations of 1%w/w Avicel and 1%w/w lignin, so as to have similar conditions as in the hydrolysis of the pretreated biomass and being able to assess the impact of enzyme deactivation by the lignin. However, due to the higher digestibility of pure cellulose, the enzyme dosing was decreased to 10 FPU g^{-1} cellulose. A control experiment without lignin addition shows that Avicel itself is well digestible with a glucose yield of 96% after 120 h (Fig. 8). Even though all lignin residues inhibited the hydrolysis of Avicel to some extent, the inhibition by the residual lignins from samples treated with low concentrations of 2-naphthol (0–4%w/w) was particularly remarkable. The glucose yield after 120 h was decreased up to 87% relative to the yield from pure cellulose due to the presence of lignin. The very small progress in the hydrolysis of these samples between 4 and 120 h indicates that the enzyme deactivation occurs very rapidly and enzymes bind quickly to lignin. The least repolymerised lignin decreased glucose yields only by 32% after 120 h, demonstrating the high impact of repolymerised lignin structures on enzyme deactivation.

Surprisingly, samples treated with 0 to 4%w/w 2-naphthol inhibited the Avicel hydrolysis similarly. A role in this effect might play, as mentioned before, a restricted accessibility of inner lignin structures in more repolymerised lignin samples or other factors which are still unknown.

Influence of lignin repolymerisation on its nanostructure and the consequences for enzyme deactivation. Why enzymes seem to have an increased binding affinity to repolymerised lignin structures cannot be ascertained from the previous

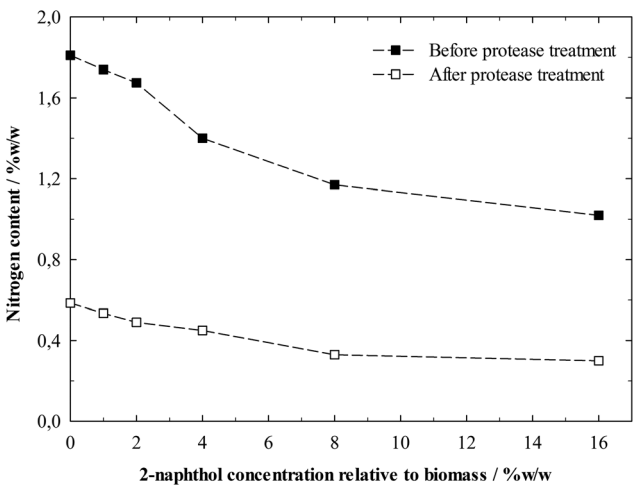

Fig. 7 Nitrogen content in enzymatically isolated lignin residues of spruce after a pretreatment with different concentrations of 2-naphthol. Nitrogen contents before and after protease treatment of the residues are shown.

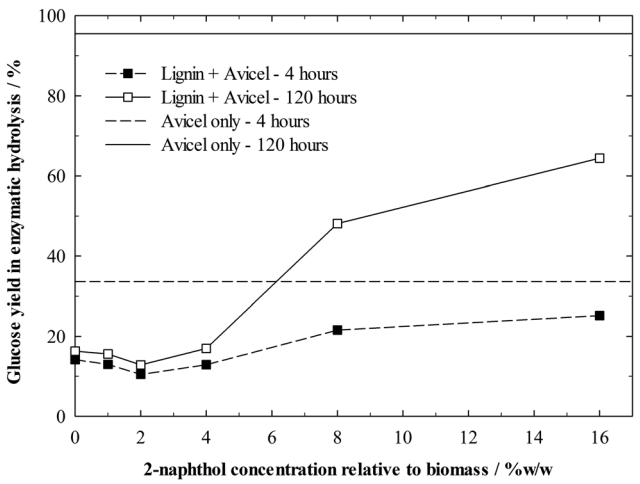

Fig. 8 Glucose yields in the enzymatic hydrolysis of Avicel after 4 and 120 h in the presence of lignin residues isolated from biomass pretreated with different concentrations of 2-naphthol. Horizontal lines represent glucose yields of the control (no lignin, only Avicel). Hydrolysis conditions: 1%w/w Avicel, 1%w/w lignin, 10 FPU g^{-1} cellulose.

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experiments and even the fundamental reasons for cellulases binding on lignin are yet to be elucidated.[59] Several authors have reported that hydrophobicity plays an important role in enzyme–lignin interactions[3,4,50] with possible contributions from electrostatic interactions.[13] The treatment with BSA, which covers hydrophobic lignin surfaces, greatly increased hydrolysis yields reflecting the importance of hydrophobic effects. It is supposed that hydrophobic interactions between the carbohydrate binding module (CBM) of the enzyme and lignin are a key driver in the adsorption process.[59]

Steam pretreatment has been suggested to increase lignin hydrophobicity due to the loss of hydroxyl and carboxylic acid functions, increasing the non-productive binding of cellulases to lignin in that way.[13] However, recent studies with lignin isolated from spruce wood did indeed observe an increased enzyme binding capacity after steam pretreatment, but could not observe an increase in hydrophobicity.[59] At the same time, a 57% increase in lignin molecular weight was observed due to repolymerisation in the steam pretreatment, which calls an influence of repolymerisation on hydrophobicity into question. This is consistent with results from this work, which showed that suppressing lignin repolymerisation did not have an influence on functional groups detectable by FTIR (Fig. S11†). The degree of lignin hydrophobicity is largely determined by its functional groups, that is to say the present study did not reveal an influence of repolymerisation on hydrophobicity neither. 2-naphthol itself has a very hydrophobic character, so its introduction into the lignin structure which was disclosed before (compare Fig. S11†) would rather be disadvantageous from this point of view. Resorcinol is very hydrophilic, still, its introduction into the lignin structure decreased yields in hydrolysis after all (compare Fig. 4). All things considered, other factors besides hydrophobicity should be taken into account when explaining the deactivating effect of repolymerised lignin on enzymes.

It is also possible that lignin repolymerisation influences lignin–enzyme interactions through modification of the physical lignin structure. This is strongly suggested by the BET analysis of the isolated lignins' specific surface areas (Fig. 9). Samples treated with low concentrations of 2-naphthol show a significantly higher specific surface area and the use of 16%w/w 2-naphthol could decrease the specific surface area by five times compared to the control. The lignin repolymerisation did considerably increase its specific surface area, which generates more potential for enzymes to adsorb and very likely accounts for the poorer digestibility in enzymatic hydrolysis.

Further insight into the modification of the physical lignin structure can be gained from the BJH analysis of the lignin pore volume distribution (Fig. 10). Higher 2-naphthol concentrations distinctly increased pore diameters, shifting the distribution maxima from less than 10 nm (control) to about 30 nm (16%w/w 2-naphthol). The BJH analysis as well provides information on the specific pore volume, shown in Fig. 9. A maximum can be found at a concentration of 2%w/w 2-naphthol, but higher concentrations steadily decreased the specific pore volume of the samples up to 44% of the control.

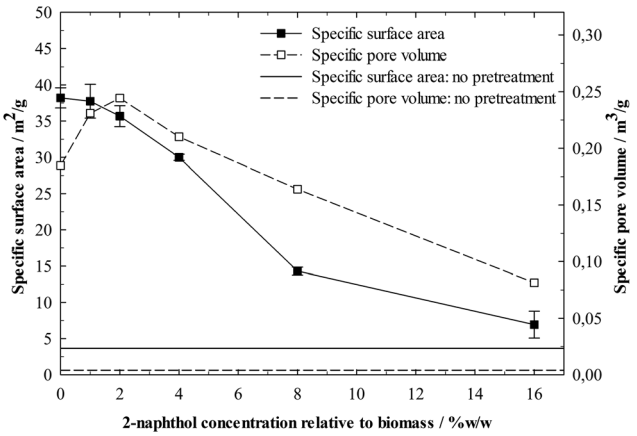

Fig. 9 BET specific surface area and BJH specific pore volume of isolated lignin residues from spruce after a pretreatment with different concentrations of 2-naphthol. Horizontal lines represent specific surface area and pore volume of lignin isolated from non-pretreated spruce.

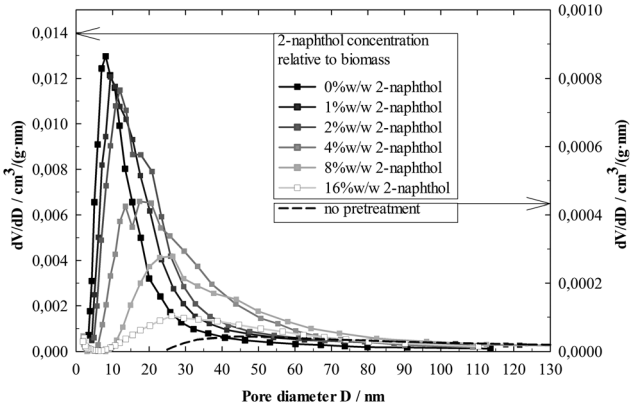

Fig. 10 BJH pore volume distribution analysis of isolated lignin residues from spruce after a pretreatment with different concentrations of 2-naphthol. The pore volume distribution of lignin isolated from non-pretreated spruce is shown as well (secondary axis of ordinate).

Though no information is available on the actual pore forms (BJH analysis assumes cylindrical pores), the decrease in specific pore volume together with the increase of the average pore diameter indicates that also the number of pores is decreased with high concentrations of 2-naphthol. Summing up, lignin repolymerisation seems to lead to smaller and also to an increased number of pores.

The mechanisms that lead to the formation of those nanostructures have still to be elucidated. The wood cell wall has a complex hierarchical organisation, starting with cellulose microfibrils of 3 nm that group together to form macrofibrils 20–30 nm in diameter.[60] Those macrofibrils are embedded in hemicellulose and enwrapped by lignin.[61] BET specific surface area measurements of pretreated but non-hydrolysed biomass gave very low values <1 $m^2\ g^{-1}$. Not until cellulose was removed in the lignin isolation procedure, higher specific

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surface areas between 7 and 38 $m^2\ g^{-1}$ (Fig. 9) were measured, revealing a matrix of lignin structures in which the cellulose fibres were embedded and which seems to be modified during the pretreatment. Lignin that had not underwent a pretreatment and was isolated from raw spruce by ball milling and enzymatic hydrolysis, showed a very low specific surface area and pore volume (Fig. 9) and comparatively high pore diameters (Fig. 10). This indicates that the observed porous lignin structures with small pores are formed during the pretreatment.

The BJH analysis of the most repolymerised lignin (0%w/w 2-naphthol) revealed pore structures with diameters smaller than 4 nm, so smaller than the diameter of typical macrofibrils. This suggests the possibility of lignin cross linking (*e.g.* through repolymerisation) occurring within or crossing the cellulose macrofibrils (compare Fig. 11). In that way, an advancing cross linking could lead to a more "fine meshed" network of the lignin matrix, which could explain the simultaneous decrease in pore size and increase in specific surface area as observed in samples pretreated with low concentrations of 2-naphthol. Another (contrary) approach to explanation is based on the coalescence of lignin, *e.g.* through physical condensation. It has been reported that in autohydrolysis and acidic pretreatments at temperatures above the lignin glass transition temperature, lignin can coalesce and migrate into the bulk liquid phase, resulting in lignin redeposition in the form of droplets on the cell wall surface that have a negative impact on cellulose hydrolysis.[62–64] Lignin coalescence might also lead to the modification of the lignin matrix and the formation of porous structures as observed in this work, which were found to have a negative impact on hydrolysis as well.

SEM analysis of the lignin residues did indeed reveal coalesced/condensed-like structures. The sample pretreated without 2-naphthol showed numerous droplets which were dispersed all over the biomass surface (Fig. 12a, further images provided in Fig. S15a†) and certainly had a negative impact on hydrolysis. The droplets had a broad size distribution ranging from around 30 nm up to several μm. In contrast, the sample pretreated with 16%w/w 2-naphthol exhibited much less of those structures (Fig. 12b, further images provided in Fig. S15b†). The presence of spherical ball-shaped structures can be observed in both samples and is regarded as typical for the redeposition of lignin from the liquid phase on the biomass.[62,64] It is also conceivable however, that the observed structures were formed when hydrophobic lignin emerges from the "inner" of the biomass upon contact with the bulk aqueous phase. It remains to be clarified as well if such structures are formed by physical coalescence or chemical repolymerisation reactions. In every way, the modification of the physical lignin structure seems to play the major role in explaining the effect of 2-naphthol on enzymatic hydrolysis.

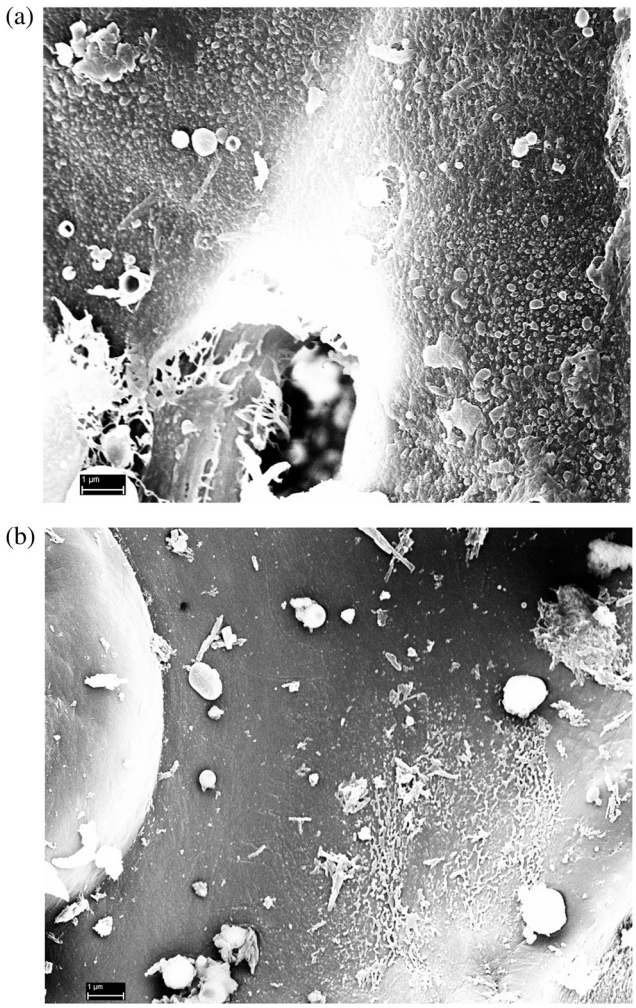

Fig. 12 SEM images of lignin residues isolated from spruce pretreated without (top) and with 16%w/w 2-naphthol relative to biomass (bottom).

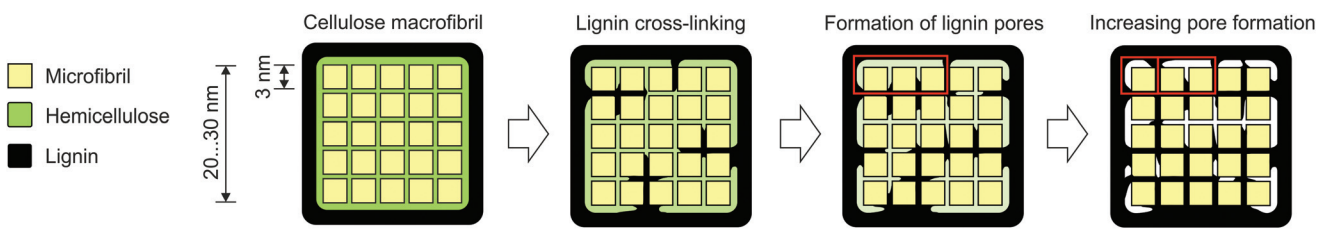

Fig. 11 Scheme denoting a possible way for the formation of porous lignin structures by cross-linking within a cellulose macrofibril during pretreatment. Advancing cross-linking increases the amount of pores while decreasing their average diameter (heighted by red squares).

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4. Conclusions

The high recalcitrance of softwood to processing by enzymatic hydrolysis seems to be of a twofold character. In the first place, autohydrolysis pretreatments with very high severities or acidic pretreatments are needed to obtain a highly accessible cellulose. On the other hand, those harsh pretreatment conditions will always be accompanied by extensive lignin repolymerisation. The lignin repolymerisation seems to modify its nanostructure in a way that significantly increases its specific surface area. This poses an additional obstacle to hydrolysis, as it increases the binding and deactivation of enzymes. The prevention of lignin repolymerisation by carbocation scavengers in such pretreatments can help to overcome this problem and allow for the required harsh pretreatment conditions. Next to increasing the biomass digestibility, such a process could add value to the obtained lignin fraction by avoiding lignin-lignin repolymerisation reactions that render these products nearly impossible to upgrade. An improved valorisation of both cellulose and lignin seems possible by inhibiting lignin repolymerisation in the first stage of most biorefinery concepts – the pretreatment.

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